THE UNIVERSITY
of EDINBURGH

Exploring the Role of the Phosphatidylinositol-3'-Kinase (PI3K) Pathway in Primordial Follicle Activation and Subsequent Development.

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Ph.D.

UNIVERSITY OF EDINBURGH

2016

Declaration

I declare that this degree has been my own work and has not been submitted for any previous degree. The work described is my own work and all work of other authors is duly acknowledged. I have also acknowledged all assistance given to me during the course of these studies.

Susan Claire Spence

Abstract

Mammalian females form their germ cells (oocytes) before or shortly after birth. The oocytes interact with somatic cells to form primordial follicles, creating the quiescent population from which oocytes will be recruited to grow throughout life. A female's fertility life span is therefore, dependant on the size of this pool and the rate at which primordial follicle are activated to grow. However, there is still much we do not know about the quiescent follicle population and the mechanisms that control their recruitment into the growing follicle population are still unclear. There is evidence that the phosphoinositide-3-kinase (PI3K) pathway is key to activation of follicle growth. The role of the PI3K pathway has been primarily explored in the rodent model and has highlighted this pathway's importance both in the activation of quiescent follicle growth and maintaining dormancy of the quiescent follicle population. This thesis aimed to explore if the PI3K pathway played a similar role in a large mono-ovulatory species as it does in the small polyovulatory rodent species.

Bovine is a mono-ovulate species, which has similar attributes in its reproduction and folliculogenesis to the human *in vivo*; therefore using an *in vitro* bovine model might be a valuable indication of the role of the PI3K pathway in the human. Initial experiments tested if the bovine was a good model for human primordial follicle activation in an *in vitro* environment. It was observed that the bovine and human had comparative levels of activation and subsequent increases in both the primary and secondary follicle populations within an *in vitro* culture system. These similarities indicate that the bovine is a relevant model for the human *in vitro*.

It is not possible to culture the entire bovine ovary. Therefore knowing the location of the primordial follicles is important to establishing what region(s) of the ovary to use. The overall concentration of ovarian follicles was higher in the cortex and gradually declined through the consecutive inner layers of the ovary. The distribution of the ovarian follicle populations were different in each distinctive region of the ovary with the quiescent follicles representing a much larger proportion of the ovarian follicle population in the cortex compared to the inner regions of the ovary. The location an ovarian follicle in the ovary was seen to influence its health in both the quiescent and growing follicle populations, with reduced health seen in the

inner layers of the ovary compared to the cortex. This resulted in very few healthy quiescent follicles outside of the cortex region making it the more favourable region to culture in functional studies.

The role of the PI3K pathway was therefore explored using an *in vitro* bovine model using the pharmacological compounds bpV (HOpic) and 740Y-P, both of which caused an up-regulation of the PI3K-pathway. It was observed that up-regulation of the PI3K pathway caused an increase in the activation of the quiescent follicle population, and the resulting primary follicles were larger in size. However, there was reduced health in both the growing and quiescent follicle populations. The ill health appears to be due to a disruption in the co-ordination of growth between oocyte and granulosa cells in the ovarian follicles, leading to enlarged oocytes in both the primary follicles and quiescent follicles. Although the PI3K pathway caused an increase in quiescent follicle activation and larger primary follicles there was no increase in the number of viable large secondary follicles obtained. The growth of the secondary follicles was unaltered by the initial activation of the quiescent follicles via the PI3K pathway. These experiments show that the PI3K pathway plays a role in primordial follicle activation in large mono-ovulate species. However, up-regulating the PI3K pathway results in a decrease in health of the quiescent and primary follicle populations, thus limiting its immediate value as a therapeutic target.

This study has improved our understanding of the role of the PI3K pathway in primordial follicle activation in a large mono-ovulate species. It has highlighted that the up-regulation of the PI3K pathway using both bpV (HOpic) and 740Y-P increases the activation of the bovine ovarian follicles *in vitro*. However, up-regulating the PI3K pathway disrupts the development of the ovarian follicles resulting in retarded growth and thereby a decrease in the survival of both the quiescent and growing follicle populations. The similarities in activation, growth and development between the bovine and the human *in vitro* indicate that the results observed in the bovine are a good indication of what would occur in the human. This study has also improved our understanding of the location, distribution and viability of the ovarian follicle population within the ovary.

Presentations Presented from this Thesis

Abstracts of oral presentations

Spence SC, Anderson RA, Telfer EE.

The role of the phosphatidylinositol-3'-kinase (PI3K) pathway in primordial follicle activation and early ovarian follicle growth.

Society for the Study of Reproduction 2014

Spence SC, Anderson RA, Telfer EE.

The role of the PI3K pathway in bovine primordial follicle activation and the regulation of ovarian follicle growth *in vitro*

ESHRE 2013

Abstracts of poster presentation

Spence SC, Anderson RA, Telfer EE. The role of the phosphatidylinositol-3’-kinase (PI3K) pathway in bovine follicle development *in vitro*.

Society for the Study of Reproduction 2013

Acknowledgements

I would like to start by thanking my supervisors Evelyn Telfer and Richard Anderson who have both been very supportive thought out my PhD. I would also like to thank Marie McLaughlin and Rosemary Bayne who took the time to teach me many of the experimental techniques I used in PhD and for the advice they have given over the years. I would also like to thank John Binnie who collected the bovine ovaries from the abattoir. Thank you to Joan Creiger and Anne Saunderson who collected the human ovarian sample. I wish to thank the University of Edinburgh and the Medical Research for funding my PhD. I also wish to thank my parents, sister and partner who have been very encouraging and supportive throughout my PhD.

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List of Abbreviations

AMH	anti-mullerian hormone
BMP	bone morphogenetic proteins
bFGF	basic fibroblast growth factor
FSH	follicle-stimulating hormone
Foxo3a	forkhead box 03a
GDF9	growth differentiation factor-9
GDNF	glial-derived neurotrophic factor
LH	luteinizing hormone
KGF	kerinocyte growth factor
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NGF	nerve growth factor
p85	regulatory subunit of phosphoinositide-3-kinase
p110	catalytic subunit of phosphoinositide-3-kinase
PD	postnatal day
PDGF	platelet-derived growth factor
PDK1	3’-phosphionositide-dependent kinase 1
PGCs	primordial germ cells
PI3K	phosphoinositide-3-kinase pathway
PKB	protein kinase B (Akt)
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-3,4,5-triphosphate
POF	premature ovarian failure
POI	primary ovarian insufficiency
PACAP	pituitary adenylate cyclase-activating polypeptide
PTEN	phosphatase and tensin homolog
rpS6	ribosomal protein S6 kinase
RPTK	receptor protein tyrosine kinases
SH2	Src homology

Tsc	tuberlin/tuberous sclerosis
ZP	zona pellucida

Chapter One:
Introduction

1.1 General Background

The mammalian ovary is the central organ within the female reproductive system. It has a number of key roles including the production of sex steroid hormones that regulate the development of secondary sex characteristics, and preparing the uterus and fallopian tubes for fertilisation and pregnancy. In addition to its role as an endocrine organ the ovary contains the supply of female germ cells (oocytes) contained within ovarian follicles. In the mammalian ovary the store of oocytes are held in a dormant state in primordial follicles, these oocytes are arrested at the diplotene stage of the first meiotic prophase and are considered to be immature as at this stage they can not be fertilised (Escobar et al., 2011; McGee and Hsueh, 2000, Telfer, 1998). In order for a mature oocyte to be produced the primordial follicles must undergo folliculogenesis. Folliculogenesis is the progression of the primordial follicles to growth and develop to become primary, preantral (secondary), antral and eventually pre-ovulatory follicles. Progression through the different developmental stages by the ovarian follicle allows its oocyte to grow and develop enabling it to reach a stage where the oocyte can resume meiosis, at this point the oocyte is considered to be mature as it possible for the oocyte to be fertilised. Ovarian follicles undergoing folliculogenesis make up the growing follicle population in the ovary (Binelli and Murphy, 2010; McGee and Hsueh, 2000).

1.2 Structure of the Ovary

The structure of the ovary varies widely between different species. However, within most mammalian species it is possible to identify three distinct structures in the ovary; surface epithelium, cortex and medulla. The surface epithelium is the outer most layer of the ovary and is made of a single layer of cuboidal cells. The medulla region is found in the centre of the ovary and is formed from loosely connected connective tissue and is highly vascularised (Escobar et al., 2011). Between the surface epithelium and medulla lies the cortex comprising of stromal

tissue formed from tightly compact connective tissue and is a poorly vascularised region.

The ovarian follicle population is not evenly distributed in the ovary since around 90% of the entire store of ovarian follicles, at all stages of their development, are located in the cortex (Escobar et al., 2011; vanWezel and Rodgers, 1996). In the cortex the ovarian follicle population is concentrated at the outer cortex and gradually declines through the consecutive layers of the ovary from the inner cortex to the cortex-medulla border and finally to the medulla itself. The location of the ovarian follicles appears to be influenced by their developmental stage, with primordial follicles predominately found in the outer cortex, whereas, growing follicles are mainly found in the inner cortex and the cortex-medulla border (Jimenez, 2010; vanWezel and Rodgers, 1996) (see figure 1.1).

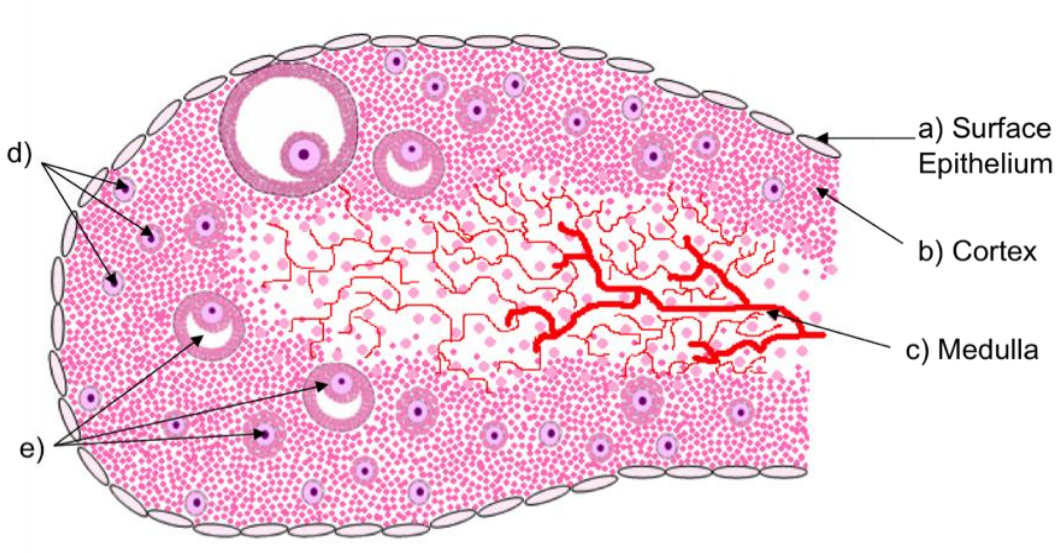


Figure 1.1: Structure of the Ovary. The ovary consists of the a) surface epithelium at the outer edge of the ovary. The next layer into the ovary is b) cortex and then at the center is c) medulla. Here it is possible to see that d) smaller non-growing follicles are found pre-dominantly in the outer regions of the cortex and e) growing follicles are found mainly in the inner cortex and at the cortex-medulla border.

In current clinical practice for fertility preservation (von Wolff et al., 2009) and *in vitro* research examining primordial follicles (Braw-Tal and Yossefi, 1997; McLaughlin and Telfer, 2010; Telfer et al., 2008; Wandji et al., 1996a; Wandji et al., 1997) in species where culturing the whole ovary is not possible due to its large size

(Telfer et al., 2000; Thomas et al., 2003; Wandji et al., 1996b) only the cortex is utilised as this is where the primordial follicles are predominately thought to be located. However, a recent study by Kristensen et al., 2011 in the human observed that the medulla did not only contain a population of growing follicles but also contained a population of primordial follicles (Kristensen et al., 2011). This, therefore, raises the question that by only using the cortex are we losing a potential source of primordial follicles in the discarded medulla? This thesis aimed to better understand the primordial follicle population. Therefore, it is important to understand their location in the ovary and so this thesis aimed to explore the location of the ovarian follicles in the ovary and examine if their location in the ovary had any influence on the distribution of non-growing and growing ovarian follicles and their viability.

1.3 Formation of the Primordial Follicle Pool

In the mammalian ovary the formation of oocytes begins during fetal life; they are derived from a population of cells called primordial germ cells (PGCs). The PGCs are formed in early embryogenesis in the embryonic hindgut in the proximal epiblast and must migrate to colonise the gonadal ridges (early gonads), which are located alongside the ventral side of the mesonephros (Molyneaux et al., 2001; Pepling and Spradling, 2001). During this migration through the fetus the PGCs are continuously and rapidly dividing by mitosis. This rapid proliferation means that there is much great number of cells being produced than lost through cell death, allowing the PGCs to reach their species-specific number (Ginsburg et al., 1990; McLaren, 2003; Tam and Snow, 1981). This species-specific number is thought to ensure the required number of primordial follicles is produced later to ensure a normal reproductive lifespan. The proliferation and survival of the PGCs is under the regulation of a number of different factors, for example; cAMP-mediate mechanisms (De Felici et al., 1993; Dolci et al., 1993), pituitary adenylate cyclase-activating polypeptide (Pesce et al., 1996) and growth factors including stem cell factor (SCF), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF) and kit ligand (KL)

(De Felici and Dolci, 1991; Cheng et al., 1994; Godin et al., 1991; Matasui et al., 1991; Kawase et al., 2004; Takeuchi et al., 2005).

PGCs undergoing mitotic divisions in the ovary are now known as oogonia. Cytokinesis is incomplete during these divisions, which results in the oogonia still being connect by cytoplasmic bridges thereby, causing them to be clustered together to form nests (or germ cell cysts) in the developing ovary (Anderson et al., 2008; Greenbaum et al., 2011; Molyneaux et al., 2001; Pepling and Spradling, 1998; Pepling and Spradling, 2001). Eventually, the oogonia will stop dividing by mitosis and begin to enter meiosis to become primary oocytes (De Felici et al., 2009). Meiosis in the primary oocytes is arrested at the diplotene stage of meiotic prophase I (Telfer, 1998). If the oocyte is to survive it must form associations with somatic cells (pre-granulosa cells) to form a structure known as a primordial follicle (Pepling, 2012; Pepling and Spradling, 2001).

Primordial follicles represent the pool of germ cells that must last a female throughout her reproductive life. It is widely accepted that this pool is the only source of fertilisable oocytes produced, with no additional primordial follicles being created at any other point in a female's life (McGee and Hsueh, 2000; Zuckerman, 1951). However, this dogma has recently been challenged by the discovery of germline stem cells within the adult mouse and human ovary which could have the potential to replenish the primordial follicle pool (Johnson et al., 2004; White et al., 2012; Zou et al., 2009). Nonetheless, further investigation is required to better understand the role of the germline stem cells in the ovary and the reproductive lifespan.

1.4 Primordial Follicle Fate

Once the pool of primordial follicles has been established the primordial follicles have three distinct fates; dormancy, activation or death. Primordial follicles are able to remain healthily in a dormant state for years or decades depending on the species. However, each primordial follicle will eventually either be activated to undergo folliculogenesis or be lost directly from the primordial follicle pool through programmed cell death processes known as atresia (Broekmans et al., 2007; Hansen

et al., 2008; McGee and Hsueh, 2000). Activation is irreversible, therefore, once a primordial follicle is activated it must continue through the various stages of folliculogenesis or it will undergo atresia (Elvin and Matzuk, 1998). Controlling the number of primordial follicles that are activated or lost from the primordial follicle pool is therefore, key to maintaining a healthy reproductive lifespan. If either activation or loss occurs at too rapid a rate the primordial follicle pool will be exhausted faster and the individual will undergo premature ovarian failure (POF).

1.4.1 Mechanisms Involved in the Control of Primordial Follicle Fate

The mechanisms involved in primordial follicle activation and loss are still unclear although recently a number of different studies have highlighted a range of factors that appear to play a role in both activation and maintaining the dormancy of the primordial follicle population. The evidence for these mechanisms being involved in primordial follicle activation has predominately been discovered through the use of rodent models, so the next section will focus on what has been found using these models (Adhikari et al., 2013; Cecconi et al., 2004; Kim, 2012; Monget et al., 2012; Pangas, 2012; Reddy et al., 2010).

1.4.1.1 Phosphoinositide-3-kinase (PI3K) Pathway

1.4.1.1.1 The Mechanisms of the PI3K Pathway

The PI3K pathway is known to play an important role in cellular survival, growth and proliferation through a variety of downstream mechanisms (Cantley, 2002; Stokoe, 2005). It is stimulated through extracellular signalling by a range of factors, such as growth factors and hormones, binding to the receptor protein tyrosine kinases (RPTK) (see figure 1.2). This stimulates RPTK to autophosphorylate itself enabling it to bind to PI3K (Cantley, 2002; Simpson and Parsons, 2001; Stokoe, 2005). PI3K is a heterodimer consisting of a regulatory and a catalytic subunit. The regulatory unit (p85) acts to maintain the catalytic subunit (p110) in a low-activate state. Autophosophorylated RPTK binds to the Src homology (SH2) domain of the p85 subunit thereby, preventing p85 from inhibiting the p110 subunit. p110, now in a

high activated state, can phosphorylate phosphatidylinositol-4,5-biphosphate (PIP2) to become phosphatidylinositol-3,4,5-triphosphate (PIP3). The amount of PIP3 produced is regulated by phosphatase and tensin homolog (PTEN), which acts as a negative regulator to prevent this pathway becoming too up-regulated by dephosphorylating PIP3 back to PIP2. While PIP3 exists it can co-bind with 3'-phosphionositide-dependent kinase 1 (PDK1) to activate downstream components including; protein kinase B (PKB) better known as Akt and ribosomal protein S6 kinase (rpS6), which mediate various downstream components, such as forkhead box 03a (Foxo3a) and p27, to promote cell cycle progression, cellular growth and proliferation (Blume-Jensen and Hunter, 2001; Brunet et al., 2001; Cantley, 2002; Engelman et al., 2006; Stokoe, 2005; Vanderhyden, 2002).

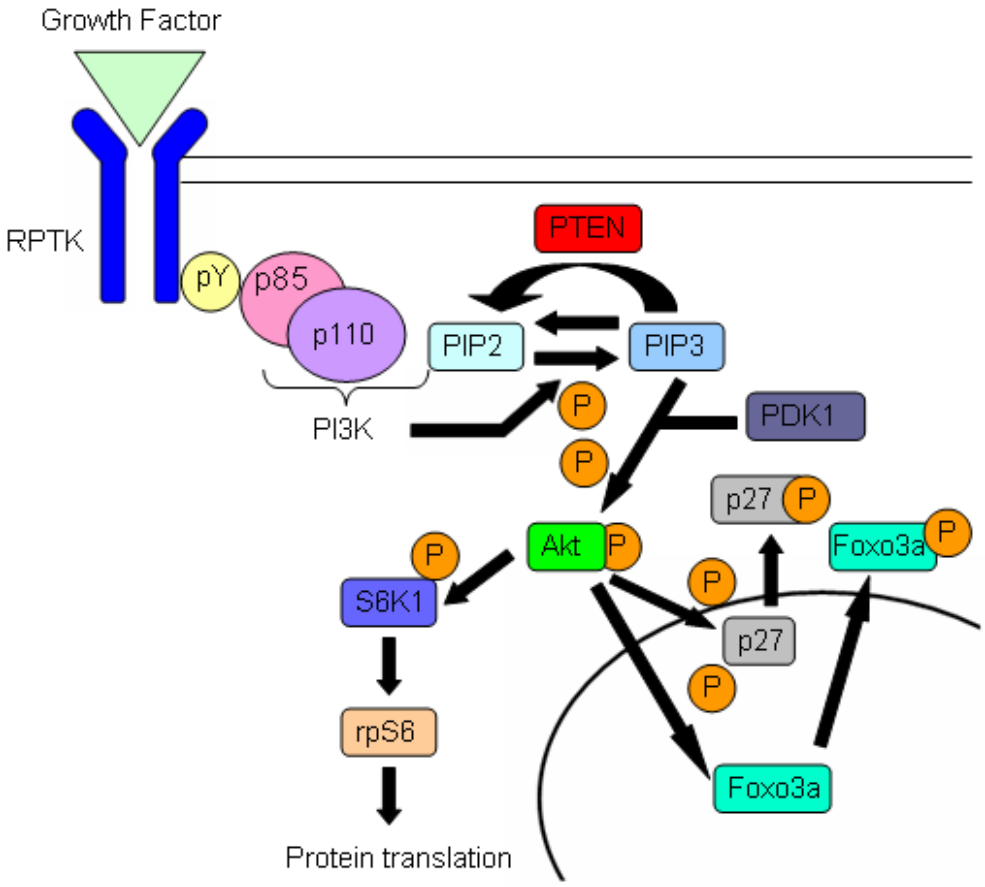


Figure 1.2: The PI3K Pathway. Stimulation of RPTK causes the activation of PI3K, which phosphorylates PIP2 to become PIP3. PIP3 then co- binds with PDK1 to activate Akt by phosphorylation. The level of PIP3 is control by PTEN, which dephosphorylates PIP3 back into PIP2. Activated Akt phosphorylates Foxo3a and p27 causing them to be moved out of the nucleus and preventing them from inhibiting cell cycle progression. Akt also phosphorylates S6K1, which leads to protein translation via rpS6, enabling cell proliferation to occur.

1.4.1.1.2 The Role of the PI3K pathway in Primordial Follicle Activation and Dormancy.

The role of the PI3K pathway in primordial follicle activation has been displayed predominately through knockout mouse models, which have targeted a number of key aspects of the pathway. The up-regulation of the PI3K pathway was explored using three knockout rodent models; PTEN knockout (Reddy et al., 2008), Foxo3 knockout (John et al. 2008, Castrillon et al 2003) and p27 knockout (Rajareddy, Reddy et al. 2007). PTEN (Reddy et al., 2008) negatively regulates the PI3K pathway by converting PIP3 back into PIP2 (Cantley, 2002). Foxo3a is part of a family of forkhead transcription factors that mediate apoptosis and cell cycle arrest through the PI3K pathway (Accili and Arden, 2004; Arden and Biggs, 2002; Brunet et al., 1999). Lastly, p27 is a cyclin-dependent kinase (cdk) inhibitor. cdk encourages the transition between the cell cycle stages therefore, p27 inhibits this to negatively regulate the cell cycle and cellular growth (Fero et al., 1996; Kaldis, 2007). Foxo3a and p27 are both only in their active state in the nucleus. Both Foxo3a (Accili and Arden, 2004; Arden and Biggs, 2002; Liu et al., 2007) and p27 (Fero et al., 1996; Rajareddy et al., 2007; Zhang et al., 1999) are located in the nucleus of the primordial follicles, whereas, the nuclei of growing follicles have no Foxo3a and the levels of p27 are increasingly reduced as the ovarian follicles progress further through folliculogenesis.

Within these three models the up regulation of the PI3K pathway did not appear to impact primordial follicle formation, as there was no change in the morphology or number of ovarian follicles compared to the control mice. However, in all of them there was a change in the rate of primordial follicle activation (Castrillon et al., 2003; John et al., 2008; Liu et al., 2007; Rajareddy et al., 2007; Reddy et al., 2008). At postnatal day (PD) 8 in oocyte-specific PTEN deficient mice the ovaries appeared to be larger in size and only 49.6% of the ovarian follicles were primordial compared to 83.6% in the control mice. By PD23 there were no primordial follicles left in the PTEN deficient mice, whereas, 69.2% of the ovarian follicles were still primordial in the control mice (Reddy et al., 2008). Similarly, the morphology of the ovaries of Foxo3a deficient mice indicates that the primordial follicles were recruited into the growing follicular pool at a faster rate, causing an increased rate of depletion in the

pool of primordial follicles (John et al. 2008, Castrillon et al 2003). Likewise, p27 deficient mice were seen to have an increase in activation, which resulted in almost all the primordial follicles being activated by the time these mice reached puberty (Rajareddy, Reddy et al. 2007). This up regulation of the PI3K pathway and subsequent increase in primordial follicle activation resulted in the mice becoming infertile at an early age. This is observed in the PTEN deficient mice having only one litter in 6-34 weeks (John et al., 2008; Reddy et al., 2008) and Foxo3a deficient mice only produced 2 litters (John et al., 2008).

The down regulation the PI3K pathway has also been explored by using knockout mouse models of; PDK1, rpS6 (Reddy et al., 2009), and a mouse model which ensured Foxo3a was constantly active (Liu et al., 2007). PDK1 is required to co-bind with PIP3 to activate Akt and thereby activating the downstream components of the PI3K pathway (Engelman et al., 2006; Mora et al., 2004). rpS6 is a downstream component of the PI3K pathway that is activated by S6K1 to promote protein translation (Volarevic et al., 2000). Lastly, Foxo3a, as mention above, is in its active state in the nuclei, where it mediates apoptosis and cell cycle arrest (Accili and Arden, 2004; Arden and Biggs, 2002; Brunet et al., 1999).

Primordial follicle formation was not impacted by a down regulation of the PI3K pathway, as none of the models show any differences in morphology or number of ovarian follicles compared to the control mice (Liu et al., 2007; Reddy et al., 2009). In the PDK1 deficient mice the pool of primordial follicles was unaltered in early life between PD8 and PD23. However, by the time the mice reached sexual maturity at PD35 the PDK1 deficient mice had only 33.7% of the number of primordial follicles seen in the control ovary. There was also a decrease in the number of activated follicles so there was only 61.6% of the proportion of growing follicles observed in the control mice. The reduction in the number of primordial and growing follicles is thought to be the reason that ovaries of the PDK1 mice are smaller in size. The primordial follicles were largely unhealthy in the PDK1 deficient mice with only 27.7% of the number of healthy follicles observed in the control ovaries (Reddy et al., 2009). Similarly, the rpS6 deficient mice had smaller ovaries and a dramatic reduction in the number of ovarian follicles by PD23. At PD35 there was a decrease in the health of both the primordial and growing follicles. By week 8 the follicle

structures had completely disappeared in the ovaries (Reddy et al., 2009). In the ovaries where Foxo3a was constantly activated there was a reduction in the number of secondary follicles and further developed ovarian follicles at both PD8 and PD13. At PD24, while wild type ovaries showed signs of ovulation, the Foxo3a activated ovaries had very few ovarian follicles reaching the later stages of follicular development and there was no evidence of ovulation having occurred (Liu et al., 2007). Both the PDK1 and rpS6 knockout mice were infertile (Reddy et al., 2009), with the primordial follicle pool being completely exhausted by 8 weeks of age in the PDK1 deficient mice (Reddy et al., 2009). In the constantly activated Foxo3a mouse model there was a dramatic reduction in fertility, as in a 6 month period only 0.3 pups were born on average per pair compared to an average of 47.4 pups per control pair (Liu et al., 2007).

Activation and growth of ovarian follicles is co-ordinated through the bi-directional communication between the oocyte and its granulosa cells (Albertini and Barrett, 2003; Elvin and Matzuk, 1998; Eppig, 2001). Interestingly, only the PTEN oocyte-specific knockout model caused an increase in primordial follicle activation (Reddy et al., 2008), whereas the PTEN specific deletion in the granulosa cells had no impact on follicle activation or dormancy (Fan et al., 2008). This, therefore, indicates that role of the PI3K pathway in the activation of the primordial follicles is controlled by the oocyte rather than the granulosa cells. This agrees with the hypothesis that it is the oocyte that has the intrinsic follicular developmental program (Eppig, 2001; Eppig et al., 2002), although further studies are required to better understand the role of both the oocyte and granulosa in primordial follicle activation.

These rodent studies therefore, show that the PI3K pathway is important for both the activation of primordial follicles and maintaining the dormancy of the primordial follicle pool. The up regulation of the PI3K pathway caused an increased rate of primordial follicle activation, whereas down regulating the PI3K pathways resulted in the primordial follicles being lost directly from the primordial follicle pool. Both resulted in premature exhaustion of the primordial follicle pool, therefore, balancing the level of PI3K pathway signalling is essential to maintain a normal healthy reproductive lifespan.

1.4.1.1.3 Pharmacological Compounds Used to Explore the Role of the PI3K Pathway.

Two pharmacological compounds bpV (HOpic) (EMO Chemical Inc., San Diego, U.S.A) and 740 Y-P (TOCRIS Bioscience, Bristol, UK) have been shown up regulate the PI3K pathway. bpV (HOpic) is a bisperoxovanadium (bpV) compound that inhibits protein tyrosine phosphatases, and is selective to PTEN. bpV (HOpic) binds to PTEN inhibiting its ability to convert PIP3 back into PIP2 (see figure 1.3) (Bevan et al., 1995; Li et al., 2010; McLaughlin et al., 2014; Posner et al., 1994; Schmidt et al., 2004). 740 Y-P is a phosphopeptide activator of PI3K. 740Y-P binds to the SH2 domain of the p85 regulatory subunit of PI3K to preventing p85 from inhibiting p110 and therefore, stimulating the activity of p110 (Derossi et al., 1998; Li et al., 2010).

740 Y-P and bpV (HOpic) cause a similar increase in primordial activation in the mouse model (Li et al., 2010) to the knockout mouse models in previous studies where PI3K pathway was up regulated (Castrillon et al., 2003; John et al., 2008; Liu et al., 2007; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008). These pharmacological compounds have allowed the role of the PI3K pathway to be explored in the human using bpV (HOpic) (Li et al., 2010; McLaughlin et al., 2014); these studies demonstrated an increase in primordial follicle activation but resulted in a decrease in follicle survival. Recently, a live human birth has been reported using bpV (HOpic) and 740 Y-P followed by replacement and IVF (Kawamura et al., 2013). In this study ovaries of 27 primary ovarian insufficiency (POI) patients were dissected into strips and exposed to bpV (HOpic) and 740 Y-P before being autotransplanted beneath the serosa of the Fallopian tubes. The patients were monitored and in 8 of the patients follicle growth was observed and at the antral stage (>5mm) of development the patients were treated with daily doses of FSH. When the follicles reached a size >16mm the patients were injected with hCG and 36 hours later the egg was retrieved. Mature oocytes were retrieved successfully from 5 of the patients and underwent intracytoplasmic sperm injection and when the embryos reached the four-cell stage they were cryopreserved so that they could be transferred back into the patient at a later date. The patient who went on to give birth

following this treatment at the age of 29 had experienced irregular cycles at the age of 23 and at 25 had become amenhorreic, with her levels of FSH seen to be elevated, however, no specific cause was found. This study suggests that stimulating Akt could be a new method to improve current fertility treatments. However, it is hard to determine the role of the PI3K pathway as this study did not have a control and the ovary being dissected and the steroid treatments would have impacted other pathways as well as PI3K. Therefore it is important to expand our knowledge of the role of the PI3K pathway, particular within the large mono-ovulatory species, which this thesis aimed to do.

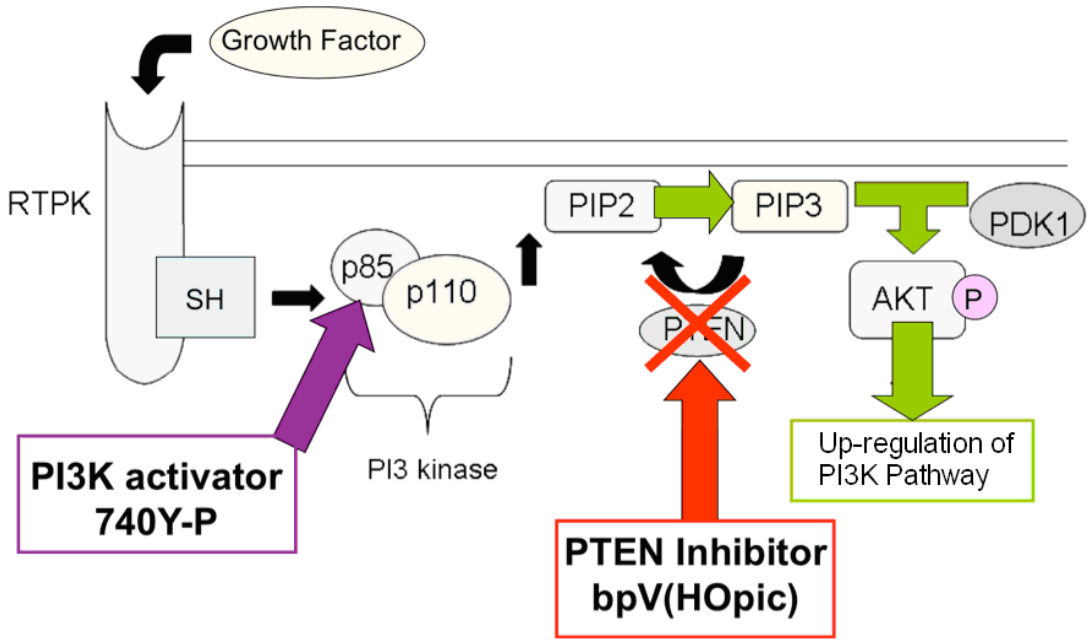


Figure 1.3: Interactions of the pharmacological compounds bpV (HOpic) and 740 Y-P with the PI3K pathway. bpV (HOpic) inhibits PTEN preventing it from converting PIP3 back into PIP2. 740 Y-P binds to the p85 subunit of PI3 kinase preventing it from inhibiting p110, resulting in increased catalytic activity of p110. Both cause the up regulation of the PI3K pathway resulting in increased levels of phosphorylated Akt and thereby, up regulate the PI3K pathway.

1.4.1.2 mTOR Pathway

1.4.1.2.1 mTORC1 Pathway Mechanisms

Mammalian target of rapamycin (mTOR) is associated with two distinct complexes known as mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) (Jacinto et al., 2004; Sarbassov et al., 2004). mTORC1 is known to promote cellular proliferation and growth by activating S6K1, thereby, inactivating eukaryotic translation initiation factors (Fingar and Blenis, 2004; Wullschleger et al., 2006). S6K1 is also responsible for the activation of rpS6 via phosphorylation, which increases protein translation and ribosomal biogenesis (Wullschleger et al., 2006). mTORC1 is negatively regulated by the tuberlin/tuberous sclerosis (Tsc) complex, Tsc1-Tsc2, through the GTPase activating protein domain of Tsc2. The role of Tsc1 is to prevent the ubiquitination and degradation of Tsc2 (Chong-Kopera et al., 2006).

1.4.1.2.2 Role of the mTORC1 Pathway in Primordial Follicle Fate

The mTORC1 pathway has been shown to be involved in primordial follicle activation through knockout mouse models, that targeted Tsc1 (Adhikari et al., 2010) and Tsc2 (Adhikari et al., 2009) of the Tsc complex. In both Tsc1 and Tsc2 oocyte-deficient mice there was an increase in mTORC1 activity and consequently its downstream components S6K1 and rpS6, showing that their deletion resulted in an up regulation of Tsc-mTORC1-p70S6 signalling.

The deletion of both Tsc1 and Tsc2 led to a shortened reproductive life in mice, which produced only two normal sized litters before becoming infertile in early adulthood. The formation of ovarian follicles was unchanged by the deletion of either Tsc1 or Tsc2 as both displayed no difference in either morphology or number of primordial follicles compared to wild type mice. By PD23 both Tsc1 and Tsc2 deficient mice were seen to have enlarged ovaries, and it was observed that all of the primordial follicles had been activated whereas, 74% of the ovarian follicles were still primordial in the controls (Adhikari et al., 2009; Adhikari et al., 2010). By 2

months the majority of ovarian follicles had become atretic, resulting in a reduced number of ovarian follicles (Adhikari et al., 2010). The primordial follicle pool was completely depleted in the Tsc1 and Tsc2 deficient mice by 3 (Adhikari et al., 2010) and 4 months (Adhikari et al., 2009) respectively, and no healthy follicular structures were found within the ovary.

Therefore, mTORC1 activity plays an important role in the activation of the primordial follicles and its level of signalling must be controlled to prevent POF. In the human the mTOR pathway was also shown to be important in maintaining the primordial follicles in their dormant state as when this pathway was down regulated, using the mTOR inhibitor rapamycin, there was a loss of ovarian follicles and a decrease in ovarian follicle health (McLaughlin et al., 2011). This indicates that mTOR is involved in controlling primordial follicle fate by regulating both their activation and maintaining their dormancy.

1.4.1.3 Interactions of the PI3K Pathway and mTOR Pathway

Activation of primordial follicles is dependent on both PI3K and mTOR signalling (Adhikari et al., 2009; Adhikari et al., 2010; Reddy et al., 2008). Although mTOR is not strictly part of the PI3K pathway it is closely related (Guertin and Sabatini, 2007; Wullschleger et al., 2006), as S6K1 is a down stream component of both signalling pathways. For S6K1 to become active it must be phosphorylated by Tsc/mTOR-p70S6 signalling at threonine (T)389 and by PI3K –Akt signalling at T229 (see figure 1.4).

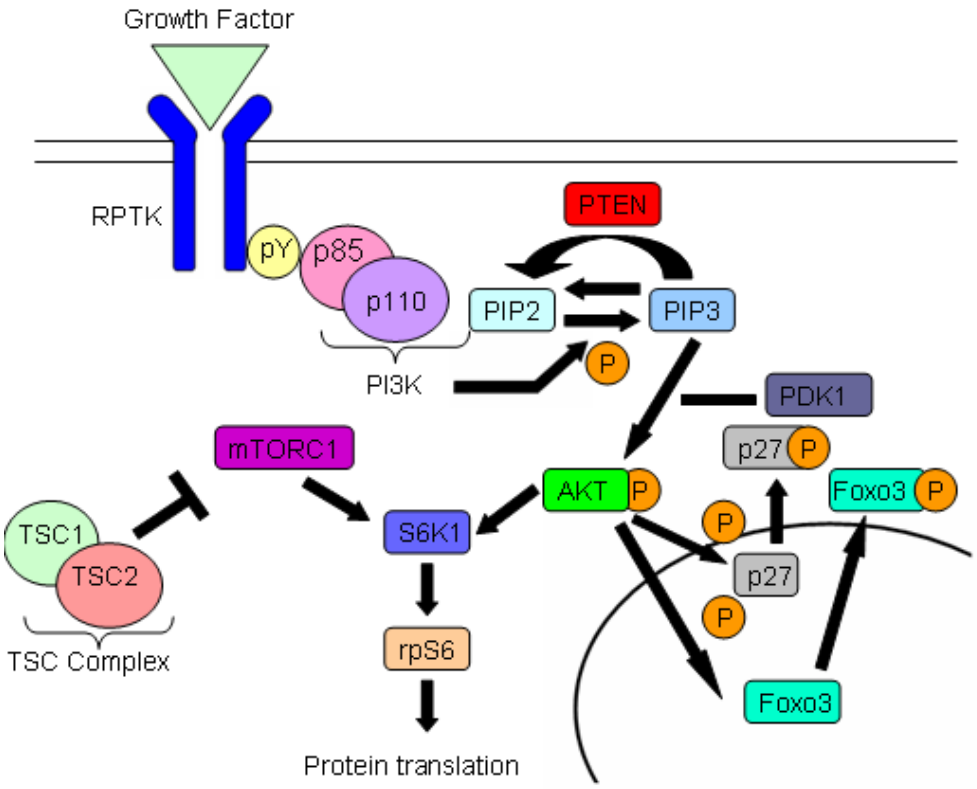


Figure 1.4: The PI3K Pathway and mTOR Pathway. The PI3K pathway and mTOR pathway are seen to converge at S6K1 as this component must be phosphorylated by both to stimulate the downstream components further on.

The pathways were shown to be independent from each other as an increase in PI3K signalling did not cause an increase in mTOR signalling (Reddy et al., 2008), and likewise, increased mTOR activity did not increase PI3K signalling. To understand the interaction between these two pathways mice deficient in both PTEN and Tsc1 were compared to mice deficient in just PTEN or Tsc1. At PD23 mice deficient in both Tsc1 and PTEN had significantly more primordial follicles activated compared to either mice with just a PTEN or Tsc1 deficiency (Adhikari et al., 2010). The interactions of PI3K and mTORC1 signalling were also examined using PTEN deficient mice in combination with the mTOR inhibitor rapamycin (Adhikari et al., 2013). At P23 there was a decrease in the size of the PTEN deficient ovaries treated with rapamycin in comparison to the untreated PTEN deficient ovaries. There were also significantly more primordial follicles in the PTEN deficient ovaries treated with rapamycin compared to the untreated PTEN deficient mice where almost the entire follicular pool had been activated. However, although rapamycin prevented all of the

primordial follicles being activated, only 20% of the ovarian follicles were primordial in comparison to 70% in the control (Adhikari et al., 2013). This indicates that mTORC1 and PI3K signalling influence one another but are able to activate primordial follicles independently.

1.4.2 The Role of RTPK Stimulation by Growth Factors in Primordial Follicle Activation.

There are a number of different growth factors which act via RPTK that have been highlighted to play a role in primordial follicle activation, including neutrophins, insulin, basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF) and platelet-derived growth factor (PDGF). The role of neutrophins was assessed through a knockout mouse model whereas; the others were assessed by exposure to varying concentrations of each growth factor.

Neutrophins are usually associated with the differentiation and survival of the neuronal population in the central and peripheral nervous system (Snider, 1994). They have also been indicated to play a role in the ovary due to their presence and the presence of their receptors (Ojeda et al., 2000) in both the oocyte and the granulosa cells (Dissen et al., 2001). An example of their role is observed in the nerve growth factor (NGF) knock out mouse model. At PD7 the NGF deficient mice had less primary and secondary follicles compared to the control where follicles at these stages were more abundant (Dissen et al., 2001). Similarly, ovaries treated with glial-derived neurotrophic factor (GDNF) had an increased number of growing follicles at day 4 with 54.3% of the ovarian follicles activated to grow in comparison to only 41.3% in the control (Dole et al., 2008).

Similarly, insulin receptors are found predominately in the oocyte of primordial follicles (Samoto et al., 1993). A study by Kezele et al 2002 treated mice with different doses of insulin, and observed an increase in the number of primordial follicles activated. At the highest dose around 30% more primordial follicles had been activated to begin folliculogenesis (Kezele et al., 2002).

Likewise, bFGF is found in the oocytes of both primordial and primary follicles (van Wezel et al., 1995; Yamamoto et al., 1997). Ovaries treated with bFGF showed

an increase in primordial follicle activation; 40% of the ovarian follicles were primordial and 60% growing follicles in the control ovaries, in the bFGF treated ovaries only 15% of the ovarian follicles were primordial and 85% were growing follicles (Nilsson et al., 2001; Nilsson and Skinner, 2004). Activation via bFGF has also been observed in the human (Wang et al., 2014).

KGF is a prototypical mesenchymal factor (Rubin et al., 1995), which helps promote the transition of the primordial follicles into primary follicles. Rat ovarian cultures treated with KGF showed 65% of primordial follicles had been activated in comparison to the control where only 45% of primordial follicles had been activated. KGF exposure to the rat ovaries caused an increase in KitL mRNA expression and KitL exposure to the rat ovaries caused an increase in expression of KGF mRNA (Kezele et al., 2005). It is though that this is because the KGF, produced by the theca cells (Parrott et al., 1994), causes the granulosa cells to promote their KitL expression and, in a positive feed back loop, stimulates the theca cells (Parrott and Skinner, 1998).

Lastly, PDGF stimulation leads to numerous cellular responses involved in proliferation, survival and chemotaxis (Heldin et al., 1998). The PDGF protein is found within both the oocytes of the primordial and primary follicles and seen to be higher in the developing follicles. Treatment with PDGF causes a significant increase in the activation of the primordial follicles whereas, treatment with anti-PDGF neutralising antibody caused a significant decrease in primordial follicle activation (Nilsson et al., 2006).

In summary, stimulation of RTPK by growth factors such as neutrophins, insulin, bFGF, KGF and PDGF plays a role in primordial follicle activation. An increase in the stimulation of the RTPK, caused by increasing the levels of these factors, resulted in increased primordial follicle activation. When the stimulation is decreased by these factors there is a decrease in the number of primordial follicle activated.

1.4.3 FoxL2

FoxL2 is a single gene belonging to a family of winged-helix/forkhead transcription factors (Crisponi et al., 2001). In humans mutations in FoxL2 led to truncation and it is likely the individual will undergo premature ovarian failure (POF). In mice FoxL2 is expressed throughout ovarian development, in the postnatal ovary it is particularly found in the granulosa cells of primordial follicles and the concentration of FoxL2 slowly decreases as the ovarian follicle progresses further through folliculogenesis (Uda et al., 2004). In mouse models lacking FoxL2 there was normal primordial follicle formation. However, within two weeks PD very few ovarian follicles were growing follicles, and there was a lack of secondary follicles within the FoxL2 deficient ovaries. This became more defined by week 8 where the control ovary showed follicles within a range of different developmental stages whereas, in FoxL2 deficient mice almost all of the oocytes where still surrounded by a single layer of mostly flattened granulosa cells, though interestingly the oocytes appeared to be enlarged. This indicates that granulosa cell proliferation had been disrupted so they were unable to complete their transition from being flattened to being cuboidal in shape (Schmidt et al., 2004; Uda et al., 2004). FoxL2 therefore, appears to be important for somatic pre-granulosa cell differentiation required for the transition from primordial follicle to primary follicle.

1.4.4 TGF-β and Smads

A number of members of the TGF- β family have been implicated to play a role in primordial follicle activation, including anti-mullerian hormone (AMH), bone morphogenetic proteins (BMP) and growth differentiation factor-9 (GDF9) (Adhikari and Liu, 2009). AMH is expressed in the granulosa cells of activated follicles but not the pre-granulosa cells of the primordial follicles (Baarends et al., 1995) and is thought to inhibit the activation of the primordial follicles and reduce the activated follicles' responsiveness to FSH (Durlinger et al., 2001; Durlinger et al., 1999; Durlinger et al., 2002). GDF-9 is secreted by the oocytes (Erickson and Shimasaki, 2000) and its expression correlates with the primordial activation as it is only

observed in the primary and secondary follicles but is not in the primordial follicles (Dong et al., 1996; Hayashi et al., 1999). BMP-4 and BMP-7 are expressed in the theca cells and are part of the BMP system, which is known to be involved in both oocyte development and the control of granulosa cell proliferation and differentiation (Shimasaki et al., 1999).

Using a knockout mouse model of AMH it was shown that a deficiency in AMH caused an increased number of primordial follicles to be activated, thereby leaving a smaller proportion of primordial follicles at both 4 and 13 months. There was a rapid depletion of the primordial follicle pool with only 38 on average being found at 13 months in the AMH deficient mice compared to 225 in the control (Durlinger et al., 1999). A role of AMH in suppressing follicle activation was also seen in human ovarian follicles (Carlsson et al., 2006).

Similarly, exposure to BMP-4 in rat ovaries *in vivo* resulted in an increase in primordial follicle activation to 60% from 53% in the untreated ovaries. Likewise, in BMP-7 exposed rat ovaries there were significantly less primordial follicles with only 56.5% of the proportion found in the control rats, leaving a mean of 416 primordial follicles in BMP-7 exposed ovaries compared to 661.4 primordial follicles in the control ovaries. Consequently, a significant fold decrease in primordial follicles was seen in the in the BMP-7 exposed rats compared to the controls (Lee et al., 2001) and a similar result was also seen in the mouse ovary (Shimasaki et al., 1999). Culturing with anti-BMP-4 antibody *in vitro* caused a significant decrease in follicle numbers after 8 days and the follicle number continued to decrease the longer the ovaries are exposed (Nilsson and Skinner, 2003).

Exposure to increased levels of GDF-9, after 10 days resulted in a 29% reduction in the number of primordial follicles, a 30% increase in primary follicles and a 60% increase in small preantral follicles (Vitt et al., 2000). Addition of GDF-9 *in vitro* also resulted in increased activation of the ovarian follicles in cortical strips of human ovarian tissue (Hreinsson et al., 2002). The reverse of this was observed in GDF-9 null mice which were infertile. They lacked corpora lutea which appeared to be due to follicle development being disrupted at the very early stages of folliculogenesis as no secondary follicles were formed (Dong et al., 1996).

Smads are a family of signal transduction proteins that transmit TGF-β signals from the cell surface to the nucleus. Smad3 is found in the oocytes of primordial and primary follicles (Xu et al., 2002). Smad3 deficient females were classified as infertile as they were unable to produce pups. At PD2 Smad3 deficient mice had significantly fewer primary follicles. By PD18 Smad3 deficient ovaries had approximately 61% more primordial follicles and 40% fewer growing ovarian follicles compared to the control. This became even more pronounced at PD90 where there was a 2.7 fold increase in the number of primordial follicles in the Smad3 ovaries and 3 fold fewer growing ovarian follicles compared to the control (Tomic et al., 2002).

In summary, members of the TGF- β family including AMH, BMP-4, BMP-7 and GDF-9, alongside Smad3, which is part of the signalling pathway for AMH and GDF-9, have been shown to play a role both in the activation and dormancy of the primordial follicles. Low levels of AMH and increased levels of BMP-4, BMP-7 and GDF-9 cause increased primordial follicle activation. A decrease in BMP-4 and Smad3 resulted in less activation and the loss of the primordial follicles directly from the primordial follicle pool.

1.4.5 Steroid Hormones

Steroid hormones have been shown to play a role in primordial follicle activation and are also known to be involved in follicle formation (Britt et al., 2004; Kezele and Skinner, 2003). Changing the steroid hormone environment by injecting androgens or aromatase inhibitors has been shown within the fetus of both sheep and baboons to impact the size of the primordial follicle reserve later on in life (Steckler et al. 2005, Albrecht and Pepe, 2010). A number of studies have shown that treatment with steroid hormones inhibits the activation of the primordial follicles (Britt et al, 2004, Vendola et al. 1999, Kezele and Skinner 2003).

Aromatase knockout mice have a reduced number of primordial follicles. However, treatment of the aromatase knockout mice at 7 weeks old with oestradiol for 21 days reversed these consequences so that these mice were seen to have no significant difference in the number of primordial follicles in comparison to the

control (Britt et al; 2004). Similarly, when both prenatal and newborn mouse ovaries were treated with oestradiol or progesterone *in vitro*, there was a reduction in primordial and primary follicles (Kezele and Skinner 2003, Chen et al. 2007).

The role of androgen was studied in rhesus monkeys. These monkeys were exposed to androgens resulting in an increase in the activation of primordial follicles to become primary follicles shown by an increase of 50% in primary follicles in 3 days. The longer the ovaries were exposed the more primary follicles were found in the monkey ovaries with a 75% increase in 5 days and 130% increase in 10 days. It is thought that androgens increase primordial follicle activation through IGF-I signalling as the levels of IGF-I and IGF-I receptor mRNA are seen to increase (Vendola et al., 1999).

It is hypothesised that the role of steroids in primordial follicle activation is limited to just after the initial wave of follicle activation (Kezele and Skinner 2003, Fortune et al. 2013). Culturing bovine fetal tissue older than 140 days with oestradiol did not have any impact on activation. In fetal tissue that was younger than 140 days treatment with oestradiol caused an increase in primordial follicle activation (Fortune et al. 2013).

1.5 Folliculogenesis

The activation of the primordial follicles is the beginning of the process of folliculogenesis (Binelli and Murphy, 2010; McGee and Hsueh, 2000). Once activation of a primordial follicle has occurred it will either continue through its progressive developmental stages until a mature oocyte is ovulated or it will undergo atresia, which can occur at any point (Scaramuzzi et al., 2011; Webb and Campbell, 2007; Webb et al., 2004). As mention above, folliculogenesis has several key developmental stages progressing from the primordial follicle to primary, preantral, antral and finally the pre-ovulatory stage (Binelli and Murphy, 2010; McGee and Hsueh, 2000) (see figure 1.5). At each stage both the oocyte and granulosa cells of the ovarian follicles are co-ordinated to develop together to ensure the correct number of mature oocytes are ovulated depending on the species. The process of folliculogenesis appears to be similar in most species however, the length of time it

takes and both follicle and oocyte size are species dependent (Evans, 2003; Mihm and Evans, 2008).

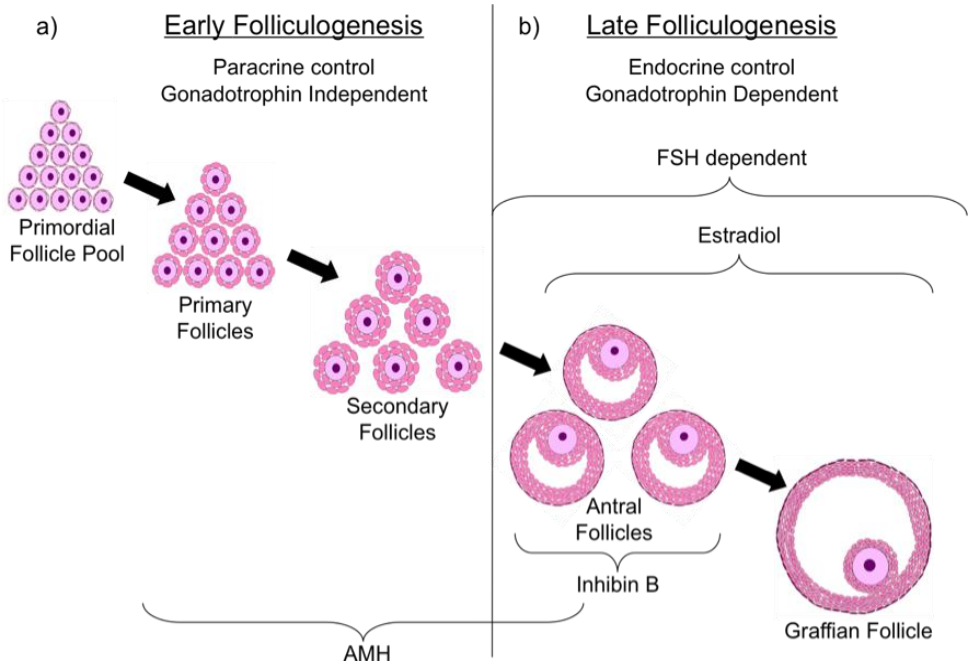


Figure 1.5: Folliculogenesis. Folliculogenesis can be separated into a) early folliculogenesis and b) late folliculogenesis. Early folliculogenesis is under paracrine control and is gonadotrophin independent whereas, late folliculogenesis is under endocrine control and is gonadotrophin dependent.

1.5.1 Early Folliculogenesis: Primordial to Preantral

Early folliculogenesis is a reference to the first few stages from the primordial stage to primary and through to the pre-antral stage of development (Binelli and Murphy, 2010; McGee and Hsueh, 2000). The difference between early and late folliculogenesis is primarily based upon how it is controlled. Early folliculogenesis is mainly under the control of paracrine factors (Kol and Adashi, 1995) and it is well established that gonadotrophins are not essential for the development of preantral follicles. This is highlighted by a number of studies where the lack of follicle-stimulating hormone (FSH) or FSH receptor had no impact on early folliculogenesis. However, there is evidence that gonadotrophins affect the development of preantral follicle in mice (Dufour et al., 1979; Edwards et al., 1977).

Folliculogenesis begins with the activation of primordial follicles. When a primordial follicle is activated there is an increase in the size of the oocyte and the flattened granulosa cells become cuboidal (McGee and Hsueh, 2000; Picton, 2001). Oocyte secreted factors GDF-9 and BMP-15 co-operate to regulate the proliferation of the cells within the follicle (Edwards et al., 2008). During the early stages of folliculogenesis GDF-9 plays the greater role in regulating the ovarian follicle cell proliferation (Dong et al., 1996; Vitt et al., 2000) whereas, in the later stage of folliculogenesis it is BMP-15 that plays the larger role (Galloway et al., 2000; Juengel et al., 2002; Juengel et al., 2004; Yan et al., 2001). The granulosa cells of the primary follicles continue to expand through mitotic divisions so the single layer of granulosa cells becomes multilayered surrounding the oocyte to form a pre-antral follicle.

During development to the pre-antral follicle stage key structural characteristics including the zona pellucida (ZP) and theca cells are formed (Knight and Glister, 2006; Oktem and Urman, 2010). These different regions of the ovarian follicle all play different parts in promoting its continuous development (Oktem and Urman, 2010). The ZP is formed from exported glycoproteins (Fair et al., 1997; Picton et al., 1998); it has a number of important roles in reproduction during oogenesis, fertilisation and implantation. However, in folliculogenesis its function is to protect the oocyte and support the communication between the oocyte and its surrounding granulosa cells (Wassarman et al., 1999). Whereas, the formation of the theca cell layer provides the ovarian follicle with an independent blood supply (Young and McNeilly, 2010), as well as being the source of androgen synthesis (Lee et al., 2004; Nilsson and Skinner, 2002).

The process of folliculogenesis depends upon coordination between all the different parts of the ovarian follicles (Canipari et al., 2012). It is generally thought that the oocyte promotes the granulosa cells to begin proliferating. However, granulosa cells are also required for oocyte growth, differentiation, meiosis and transcriptional activity (van den Hurk and Zhao, 2005). In order to have a healthy ovarian follicle the growth of the oocyte and various components of the ovarian follicle must be balanced, one factor thought to regulate this is kit ligand (KL) (Cecconi et al., 2004; Hutt et al., 2006; Otsuka and Shimasaki, 2002; Wu et al.,

2004). To maintain communication between the granulosa cells and the oocyte, physical links known as transzonal projections are formed between them. Transzonal projections stretch from the granulosa cell through the ZP to the oocyte (Combelles et al., 2004), and play an important role in ovarian folliculogenesis and are essential for maintaining the overall health of the follicle (Albertini et al., 2001; Cecconi et al., 2004; Eppig, 2001). At the oocyte membrane transzonal projections form gap junctions that are regions specialized as transmembrane channels. These gap junctions allow the exchange of ions, electrical impulses and small molecules between the oocyte and granulosa cells (Canipari et al., 2012; Cecconi et al., 2004; Fair, 2010; Grazul-Bilska et al., 1997). The transport of key components from the granulosa cells to the oocyte is required for the oocyte's growth and metabolism (Moor, 1988; Rodgers et al., 2000). The gap junctions are also thought to regulate folliculogenesis and ovarian follicle loss through atresia (Grazul-Bilska et al., 1997). Connections via gap junctions are not only found between the oocyte and the granulosa cells but also from granulosa cell to granulosa cell enabling the bidirectional communication between these different regions of the ovarian follicle to maintain the correct growth and thereby, health of the ovarian follicle (Elvin et al., 2000; Fair, 2010; Nagyova et al., 2000).

The majority of the oocyte growth is completed during early folliculogenesis before the ovarian follicle reaches the antral stage of development. During this time the oocyte increases in volume by 100-300 fold and is supported by the increasing number of surrounding granulosa cells (Griffin et al., 2006). The cytoplasm in the growing oocyte accumulates a range of substrates including glycogen granules, lipid droplets, protein and mRNA. There are also structural changes and redistribution of the endoplasmic reticulum and Golgi complexes indicating the high synthetic activity within the oocyte (Fair et al., 1997; Picton et al., 1998; Sturmey et al., 2009).

1.5.2 Late Folliculogenesis: Antral to Ovulation

Late folliculogenesis refers to the last few stages from antrum formation to ovulation (Binelli and Murphy, 2010; McGee and Hsueh, 2000). Antrum formation appears to be the crossover between early and late folliculogenesis as it is under

paracrine control, but is also influenced by gonadotrophins such as FSH (Cortvrindt et al., 1997; Monti and Redi, 2009; Zuccotti et al., 2011). However, the transition from pre-antral to antral is still primarily under the control of TFG-β ligands produced by the granulosa cell and theca cells rather than the gonadotrophins (Diaz et al., 2008). At the antral stage of folliculogenesis the follicles are gonadotrophin-dependent (Edson et al., 2009; Webb et al., 2007), with the progression through the antral stage to ovulation being dependent on the pituitary-secreted gonadotrophins FSH and luteinizing hormone (LH). Their importance is highlighted by a lack of LH receptors resulting in follicles not progressing beyond the antral stage (Zhang et al., 2001) and the lack of FSH causing folliculogenesis to stop before the antral developmental stage (Burns et al., 2001; Danilovich et al., 2000; Dierich et al., 1998). In the luteal-follicular transition there is an intercycle rise of FSH (Mais et al., 1987; Messinis et al., 1993), which allows a small cohort of antral follicles to survive atresia due to the survival actions of FSH (McGee and Hsueh, 2000). FSH accumulates in the follicular fluid in follicle recruitment and stimulates the granulosa cells to produce steroids (McNatty et al. 1975). LH stimulates the theca cells to produce androgens; which are transferred into the granulosa cells, and under the influence of FSH, are aromatized into oestrogens (Hillier et al., 1994). Androgen and oestrogen are important for the continued proliferation and differentiation of the granulosa cells (Drummond et al., 1999; Findlay and Drummond, 1999; Findlay et al., 2000).

Morphologically the antral stage of development is defined by the formation of the antral cavity (Boland et al., 1994). The formation of its antral cavity occurs when the follicles reach a size of 200-500μm again depending on the species (Picton et al., 1998) at which point the oocyte is about 80% of the final size (Durinzi et al., 1995; Szybek, 1972). Alongside the formation of the antrum there is the production of follicular fluid which is formed from components from the bloodstream and surrounding granulosa cells (Clarke et al., 2006). The follicular fluid contains many molecules including oxygen, carbohydrates, amino acids, growth factors and hormones amongst other factors (Sutton et al., 2003). The formation of the antral cavity triggers a divergence in morphology and function of the granulosa cells into mural granulosa cells and cumulus cells. The mural granulosa cells line the follicle

wall whereas, the cumulus cells are found around the oocyte (Zuccotti et al., 2011); the formation and proliferation of both are due to FSH activating the cAMP/protein kinase A pathway (Richards, 2001a).

Circulating levels of FSH appears to play the main role in controlling follicle growth and support a small group of antral follicles to grow until the granulosa cells of the largest follicle begins to produce oestradiol and inhibin. The large follicle is now referred to as the dominant follicle and it is dependent on LH, and the oestradiol and inhibin it produces suppresses FSH secretion to stop the other subordinate follicles from growing (Hennet and Combelles, 2012; Hunter et al., 2004). Although oestrogen normally suppresses gonadotrophin production, it later results in a surge of LH and FSH, this surge causes the expansion of the cumulus-oocyte complex, the loss of the gap junctions, the maturation of the oocyte and for it to be ovulated (Binelli and Murphy, 2010).

In order for the oocyte to become mature both nuclear and cytoplasmic maturation must occur so a healthy gamete is formed (Eppig, 1996). Cytoplasmic maturation refers to the development of the mechanisms needed for fertilisation, the formation of the pronuclei and the first embryonic divisions (Ajduk et al., 2008; Ferreira et al., 2009). Nuclear maturation refers to the resumption of meiosis I to metaphase II where it becomes arrested again: the oocyte is now considered to be mature. The resumption of meiosis is triggered by a surge in LH that restrains the inhibitory factors that normally prevent this from happening, resulting in the breakdown of the germinal vesicle, condensation of the chromosomes, spindle formation and the formation of the 1st polar body (Ledan et al., 2001; Mehlmann, 2005; Perry and Verlhac, 2008; Tripathi et al., 2010, Ledan et al 2001, Perry and Verlhac 2008, Tripathi et al 2010).

1.5.3 Follicular Atresia

As mentioned above the primordial follicle pool is established early in fetal life and is generally accepted as the only source of oocytes. Within the ovary hundreds of thousands of primordial follicles exist in a female at birth (Hirshfield, 1991). However, the majority of these primordial follicles will undergo atresia (Hsueh et al.,

1994; Tilly et al., 1991). For example in the human of the 600,000 – 700,000 ovarian follicles present at birth only around 400 will complete folliculogenesis to produce a mature oocyte (Block, 1953, Baker, 1963, Gougeon and Chainey, 1987, Richardson et al., 1987, Forabosco et al., 1991; Faddy et al., 1992; Faddy and Gosden, 1996).

Follicular atresia is identified through the arrested development of ovarian follicles and the beginning of degeneration and can occur at any point within the process of folliculogenesis. Degeneration impact all components of the ovarian follicles i.e. the granulosa cells, theca cells, basement membrane and the oocyte itself (Irving-Rodgers et al., 2009). Cell death pathways are key to the process of atresia and have been observed in both oocyte and the granulosa cells (Hussein, 2005; Johnson, 2003). Granulosa cell apoptosis is thought to be the main mechanism of follicular atresia (Manabe et al., 2004).

Primordial follicles are activated as a small cohort and this cohort will decrease in number as they progress through the various stages of folliculogenesis (Fortune, 1994; Macklon and Fauser, 1999), due to loss by atresia. This is thought to be one reason very few of the primordial follicles formed will ever produce a mature oocyte (Fortune, 1994; Morbeck et al., 1992).

Follicular development and oocyte development are inter-dependent and the oocyte's capability to be fertilised is dependent on how it develops through the various stage of folliculogenesis in the ovarian follicle. Oocyte quality during this process is known to be one factor that determines whether an ovarian follicle will undergo atresia or continue through folliculogenesis to become dominant and be ovulated, as advanced atresia in ovarian follicles is associated with its oocyte being of poor quality (Blondin and Sirard, 1995; Hendriksen et al., 2004; Salamone et al., 1999). Therefore the loss of follicles through atresia may be one way to ensure an oocyte of good quality is ovulated.

1.6 Folliculogenesis *in vitro*

Many studies have examined folliculogenesis *in vitro* and this has led to a number of different methods being established to grow ovarian follicles from different stages of their development including primordial, pre-antral and antral

follicular stages. These include gel droplets (Itoh et al., 2002; Loret de Mola et al., 2004; Oktem and Oktay, 2007; Wandji et al., 1996a), calcium alginate droplets (West et al., 2007; Xu et al., 2006), microporous membranes (Eppig and O'Brien, 1996; Loret de Mola et al., 2004) and v-bottom culture plates (Gutierrez et al., 2000; Thomas et al., 2007; Thomas et al., 2001), allowing the ovarian follicles to be cultured as organ cultures, in groups or as individual follicles.

It was first discovered in the rodent model that it was possible to culture ovarian follicles from the primary and secondary stage of development, *in vitro* to a point where the ovarian follicles and their oocytes had developed sufficiently enough that a mature oocyte could be collected (Cortvrindt et al., 1996; Eppig and Schroeder, 1989; Spears et al., 1994). The study used a mouse model to produce viable offspring entirely *in vitro* starting at the primordial follicle stage. The study reported the birth of a single live pup (Eppig and O'Brien, 1996). This system was later improved to increase the number of offspring produced and consisted of two steps (O'Brien et al., 2003). The first step was to culture the whole ovary of newborn mice to achieve growth from the primordial follicle to secondary follicle stage. The second step was the isolation of the oocyte-granulosa cell complexes from the secondary follicles through enzymatic dissociation, which were then further cultured to gain competent oocytes. The rate of primordial follicle activation in the mouse *in vitro* is similar to that seen *in vivo* (Eppig and O'Brien, 1996).

Unfortunately, it has not been possible to directly transfer the techniques used in the mouse to larger mammals, such as the bovine or the human, as the ovaries are too large to be cultured as whole organs. Culturing the ovary as a whole leads to anoxia, depletion of nutrients and accumulation of metabolites in the inner medulla causing necrosis. The stroma is also too tough to be able to use enzymatic dissociation since there is a risk of damaging the oocytes with this treatment (Telfer et al., 2000; Thomas et al., 2003; Wandji et al., 1996b). To avoid whole ovarian culture, which is impractical in larger mammals, systems were developed to utilise the ovarian cortex to successfully activate primordial follicles (Braw-Tal and Yossefi, 1997; Wandji et al., 1996a; Wandji et al., 1997).

The shape and density of the stroma influences the rate of activation and growth of the primordial follicles, with flattened strips showing a greater level of activation

and growth (Telfer et al., 2008), compared to those in solid cubes (Hovatta et al., 1997). The activation of primordial follicles within cortical strips allows follicles to grow as far as the pre-antral stage. However, beyond this stage of development the large portion of stroma surrounding the pre-antral follicles inhibits their ability to progress further. This therefore, led to a two-step culture system being developed in large mammals. The primordial follicles were first activated and grown in the cortical strips of the ovarian cortex and the second step was for the secondary follicles to be mechanically isolated from the ovarian tissue and further cultured individually with very little stroma, to allow the ovarian follicles to continue through folliculogenesis, although this has not yet produced a mature oocyte (Hovatta et al., 1999; McLaughlin and Telfer, 2010; Telfer et al., 2008). It is thought that a third step must be developed to aspirate and mature the oocyte-cumulus complexes which will allow the full process from primordial follicle to support oocyte maturation *in vitro* for larger mammals (Telfer and Zelinski, 2013).

1.7 Research Aims

The aim of this research is to gain a better understanding of the primordial follicle population. This will be achieved by:

- Exploring the role of the PI3K pathway in primordial follicle activation and subsequent growth and viability.
- Examining if activation via the PI3K pathway has any impact on the development of the ovarian follicles through folliculogenesis.
- Exploring the location and distribution of the ovarian follicle populations, including the primordial follicles, in the ovary and establishing if their location influences ovarian follicle health.
- Examining if the bovine is a good model of human primordial follicle activation and subsequent growth in an *in vitro* culture system.

I hypothesise that using the bpV (HOpic) and 740 Y-P to up regulate the PI3K pathway in the bovine model will cause increased primordial follicle activation. I

also hypothesise that the bovine will be a good model of human primordial follicle activation *in vitro* and that there will be a small population of primordial follicles within the medulla to use for exploring the primordial follicle population.

Chapter Two:
General Materials and Methods

2.1 Collecting Ovarian Samples

The majority of the materials and methods in this section are based upon the ovarian culture systems developed and described by McLaughlin and Telfer, 2010 and Telfer et al., 2008.

2.1.1 Bovine Tissue

Bovine ovaries were collected from the local abattoir. The animals ranged between 10-14 months in age and were in different stages of their estrous cycle. The ovaries were transported in M199 (HEPES buffered) medium supplemented with sodium pyruvate (2mM), L-glutamine (2mM), bovine serum albumin (BSA) (3mg/ml), penicillin G (75µg/ml), streptomycin (50µg/ml) and amphotericin B (2.5µg/ml) (all chemicals from Sigma Chemicals, Poole, Dorset, UK). Before being used the medium was filtered through a sterilized filter system (Corning Costar Europe, Badhoevedorp, The Netherlands) and was approximately 30-38°C on arrival at the laboratory.

2.1.2 Human Tissue

Human biopsies were obtained after informed consent from women aged between 28-40 undergoing an elective Caesarean section at local hospitals (LREC 10/S1101/24). The tissue was transported in Leibovitz medium (GIBCO BRL, Life Technologies Ltd.) supplemented with sodium pyruvate (2mM), L-glutamine (2mM), human serum albumin (HSA) (3mg/ml), penicillin G (75µg/ml) and streptomycin (50µg/ml) (all chemicals from Sigma Chemical). The samples range from 4×3×5mm to 6×4×7mm in size. Before being used the medium was filtered through a sterilized filter system (Corning Costar Europe) and was approximately 25°C on arrival into the laboratory.

2.2 Tissue Dissection in Preparation for Tissue Culture

2.2.1 Bovine Leibovitz Medium

Leibovitz medium (GIBCO BRL, Life Technologies Ltd.) was supplemented with BSA (3mg/ml), sodium pyruvate (2mM), L-glutamine (2mM), penicillin G (75µg/ml) and streptomycin (50µg/ml) (all chemicals from Sigma Chemical). Before being used this medium was passed through a sterilized filter system (Corning Costar Europe) and heated to 37°C. From here on it is referred to as bovine Leibovitz.

2.2.2 Human Leibovitz Medium

Leibovitz medium (GIBCO BRL, Life Technologies Ltd.) was supplemented with HSA (3mg/ml), sodium pyruvate (2mM), L-glutamine (2mM), penicillin G (75µg/ml) and streptomycin (50µg/ml) (all chemicals from Sigma Chemical). Before being used the medium was passed through a sterilized filter system (Corning Costar Europe) and heated to 37°C. From here on it is referred to as human Leibovitz.

2.2.3 Dissection of Bovine Ovaries to Obtain Cortical Strips

The ovarian samples the bovine ovaries were transported to the laboratory where they were placed into a laminar flow hood and rinsed in 70% ethanol. The ovaries were examined for areas where there appeared to be no growing follicles and the cortex was removed from the surface of the ovary to a depth of 1-2mm at these locations using a surgical blade (see figure 2.1). These cortical strips were then placed in bovine Leibovitz and examined under a light microscope, with a heated plate set to 37°C to maintain the temperature of the medium. The cortical strips were dissected to remove any areas which contained follicles that were 40µm or larger in diameter. Those areas without any large follicles were further dissected to a reduced size of 0.5-1 mm^2 using a surgical blade.

2.2.4 Dissection Human Biopsies to Obtain Cortical Strips

The human biopsies were placed in fresh human Leibovitz. Under a light microscope, with a heated base at 37°C, the tissue was examined and areas of medulla and areas of the cortex which had follicles of 40µm or larger in diameter were removed using a surgical blade (see figure 2.1). All areas of the cortex containing follicles with a diameter of less than 40µm were dissected to a size of 0.5-1mm^2.

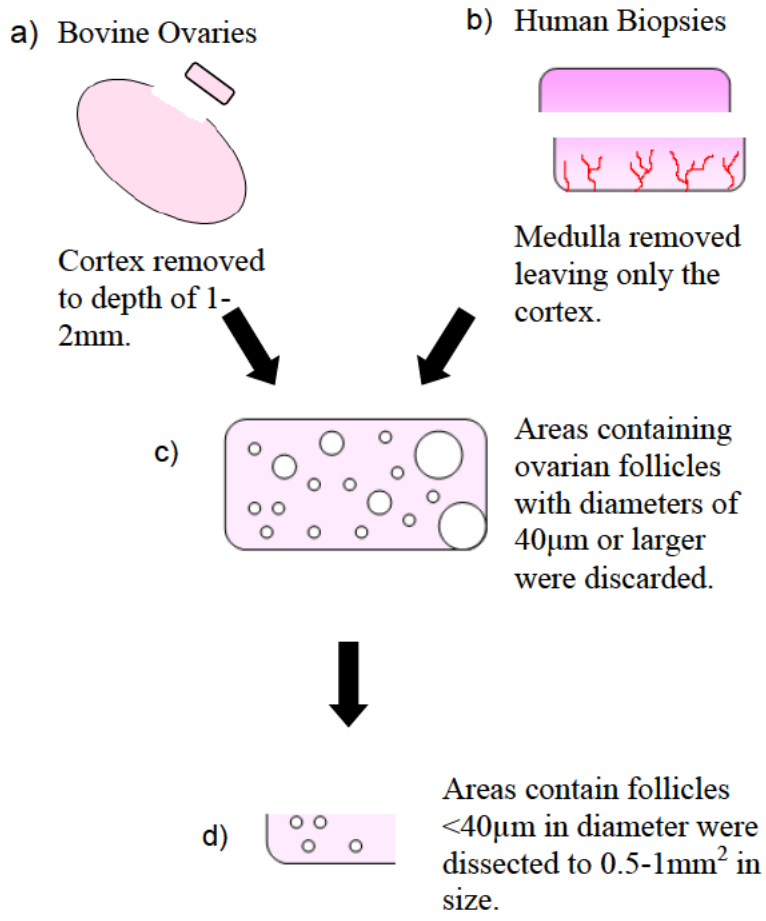


Figure 2.1 Dissection of Bovine and Human Tissue to Obtain Cortical Strips for Tissue Culture. a) Surface of the bovine ovary was collected to a depth of 1-2mm while trying to avoid large follicles. Whereas, in the b) human samples the cortex was extracted from the biopsies by removing the medulla. c) Both the bovine and human tissue fragments were examined under a light microscope and any areas containing ovarian follicles that were 40µm in diameter or larger were discarded. d) Areas with follicles less than 40µm in diameter were dissected to a size of 0.5-1mm^2.

2.5 Tissue Culture

2.5.1 Bovine McCoy's Medium

McCoy's medium (GIBCO BRL. Life Technologies Ltd.) was supplemented with L-glutamine (3mM), penicillin G (0.1mg/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), BSA (1mg/ml) and bovine insulin (10ng/ml) (all chemicals from Sigma Chemical). This medium was passed through a sterilized filter system (Corning Costar Europe) before being used. The medium was equilibrated in a humidified incubator at 37°C with 5% CO_2 and further supplemented with ascorbic acid (50µg/ml) and recombinant human (rh)FSH (1ng/ml) (Sigma Chemical) just before being used. From here on this medium is referred to as bovine McCoy's.

2.5.2 Human McCoy's Medium

McCoy's medium (GIBCO BRL. Life Technologies Ltd.) was supplemented with L-glutamine (3mM), penicillin G (0.1mg/ml), streptomycin (0.1mg/ml), transferrin (2.5µg/ml), selenium (4ng/ml), HSA (1mg/ml) and human insulin (10ng/ml) (all chemicals from Sigma Chemical). This medium was passed through a sterilized filter system (Corning Costar Europe) before being used. The medium was further supplemented with ascorbic acid (50µg/ml) and rhFSH (1ng/ml) (Sigma Chemical) and equilibrated in a humidified incubator at 37°C with 5% CO_2 just before being used. The medium from here on is referred to as human McCoy's.

2.5.3 Culturing Bovine and Human Cortical Strips

The dissected cortical strips or the tissue that had been treated with 740 Y-P or bpV (HOpic) (see section 2.6) were cultured individually in 300µl of bovine or human McCoy's based on the respective species of the tissue in 24 well plates (Corning Costar Europe) (see figure 2.2). The plates were placed into a humidified incubator at 37°C with 5% CO_2 for 6 days. Every 48 hours 150µl of the medium in

the wells was removed and replaced with 150µl of fresh bovine or human McCoy's respectively.

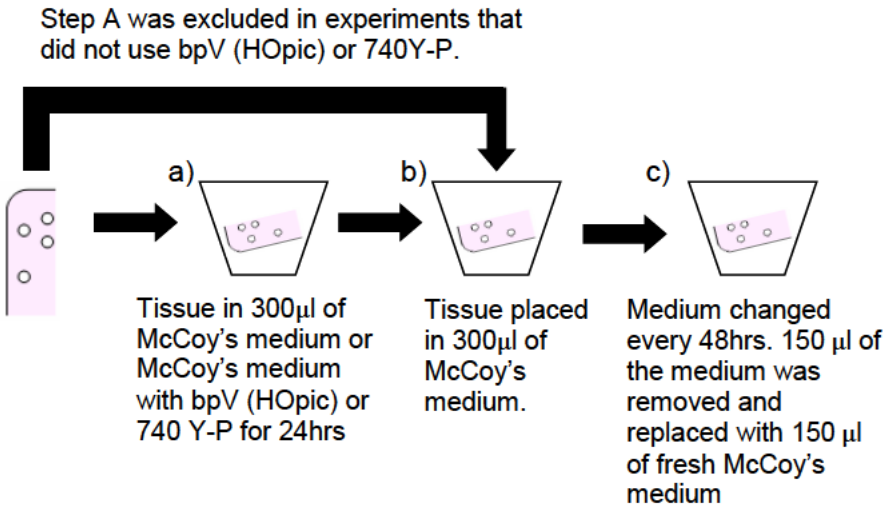


Figure 2.2 Tissue Culture. Dissected tissue fragments to be treated were placed in a) bovine McCoy's medium or bovine McCoy's media with either bpV (HOpic) or 740 Y-P at various concentrations. Treated tissue or none treated tissue was placed into b) 300µl of bovine or human McCoy's medium respectively and c) the medium was changed every 48hrs by 150µl of the medium being removed and replace with 150µl of fresh bovine or human McCoy's medium respectively.

2.6 Treatments

740 Y-P (TOCRIS Bioscience) and bpV (HOpic) (EMO Chemical Inc.) were used only in some experiments to explore the PI3K pathway. Both 740 Y-P and bpV (HOpic) are supplied in a powdered form and were dissolved then diluted using sterilized distilled water. In these experiments the dissected cortical strips were first placed in 300µl of either bovine McCoy's or bovine McCoy's with 1µM, 10µM or 100µM of bpV (HOpic) or 0.1µg/ml, 1ug/ml, 10µg/ml or 100µg/ml of 740Y-P in 24 well plates (Corning Costar Europe) (see figure 2.2). The tissue was incubated in a humidified incubator at 37°C with 5% CO_2 for 24 hours, before being further cultured in bovine McCoy's for a further 6 days as described in section 2.5.3.

2.7 Histological Processing

2.7.1 Tissue Fixation and Processing

After completing the cultures the tissue was fixed in 4% paraformaldehyde (ADAMS, Leeds, UK) for approximately 48 hours at room temperature. The 4% paraformaldehyde was then removed and replaced with increasing concentrations of ethanol; 70%, 90%, 100% for approximately 1 hour for each concentration. The tissue samples were then removed from the 100% ethanol and transferred to plastic moulds (Polyscience Inc., Warrington, U.S.A) filled with cedar wood oil (Fisher Scientific, Loughborough, Leicestershire, UK) for approximately 24 hours. The cedar wood oil was then removed and replaced with toluene (Fisher Scientific) for 30 minutes in a fume hood. The tissue was then placed in paraffin wax (McCormack Scientific, St Louis, Mo) in a 60°C oven; the paraffin wax was then changed three times into fresh paraffin wax at hour intervals. On the last wax change the tissue was embedded into the wax ensuring a thin layer of wax was between the bottom of the plastic mould and tissue. This was then placed to cool at -4°C.

2.7.2 Tissue Sectioning and Mounting

The tissue was sectioned on a microtome (Leica UK Ltd, Milton Keynes, UK) at a thickness of 6µm. Bovine tissue was placed onto polylysine slides (Thermo Scientific, Loughborough, UK) and human tissue was placed on Superfrost Plus slides (Thermo Scientific). The tissue was placed onto their respective slide type by floating the sections in a water bath at 42°C. The tissue was dried overnight in a 37°C oven.

2.7.3 H&E Staining

Sections were de-waxed in xylene for 30 mins and then placed through decreasing concentrations of ethanol at 100%, 90% and 70% then into water to rehydrate the sections. The sections were then placed into Haematoxylin (BDH

Laboratory Supplies Poole, UK) for 3.5 minutes, then rinsed under running water then briefly dipped in acid alcohol and again rinsed in running water. Sections were placed into Scotts tap water substitute for 3 minutes and rinsed in running water for 3 minutes. Sections were then placed in an eosin solution (1:1) (Sigma Chemicals) for 2 minutes then dipped into running water before being transferred into potassium aluminium solution for 3 minutes. The sections were again rinsed in water before passing through increasing concentrations of ethanol at 70%, 90% and 100% to dehydrate the sections. The sections were then placed in xylene for 15 minutes before being mounted using DPX (BDH Laboratory Supplies) and glass cover slips (BDH Laboratory Supplies).

2.8 Analysis of Follicle Development and Health in Cortical Strips

In the bovine every tenth section of the mounted cortical strips were counted to prevent double counting of the ovarian follicles, as an ovarian follicle with the germinal vesicle present would span more than one section. Only follicles that had a germinal vesicle and nucleolus were included in the analysis. In the human tissue all sections were analyzed. Each follicle was counted and assessed in the section where it had the largest diameter with the germinal vesicle and nucleolus present. The follicles were classified as quiescent, primary or secondary based on their morphology (vanWezel and Rodgers, 1996; Wandji et al., 1996b) (see figure 2.3). (1) Quiescent follicles included both primordial follicles and transitory follicles. Primordial follicles consisted of an oocyte surrounded by a single layer of flattened granulosa cells and transitory follicles had a mixture of flattened and cuboidal granulosa cells surrounding the oocyte. (2) Primary follicles consisted of a single layer of cuboidal granulosa cells surrounding the oocyte. (3) Secondary follicles had two of more layers of granulosa cells surrounding the oocyte.

Follicle health was based on: (1) general circularity of the oocyte, (2) integrity of the oolemma in the oocyte, (3) presence of a germinal vesicle and nucleolus in the oocyte and (4) that 85% of the granulosa cells were healthy. Each follicle was classified as healthy if it met these criteria and unhealthy if the follicle did not fit these criteria.

Each ovarian follicle was measured across its whole diameter at its largest and then at a 90° angle to the first measurement a second measurement was taken (see figure 2.3). The oocyte diameter were measured twice along the same lines as those used to measure the follicle diameter. The average follicle diameter and oocyte diameter was established from these two measurements.

The concentration of ovarian follicles was estimated within the cortical strips of both the human and bovine. In the bovine 6 photos were periodically taken through each of the cortical strips. Using Image J 1.44g (Rasband W.S, National Institutes of Health, USA, http://imagej.nih.gov/ij/) it was possible to calculate the volume of each of the photographed sections. The number of ovarian follicles in the photograph sections were counted and used alongside the calculated volume to estimate the concentration of ovarian follicles in each sample. In human tissue a photo was taken at every 20^{th} consecutive section. Again using Image J 1.44g (Rasband W.S, National Institutes of Health, USA, http://imagej.nih.gov/ij/) it was possible to calculate the volume of each of the photographed sections. The volume of the photographed sections amounted to 5% of each cortical strip this was used to estimate the total volume of each cortical strip. The total number of ovarian follicles was counted in each cortical strip was then used with the estimated total volume to determine the concentration of the ovarian follicles.

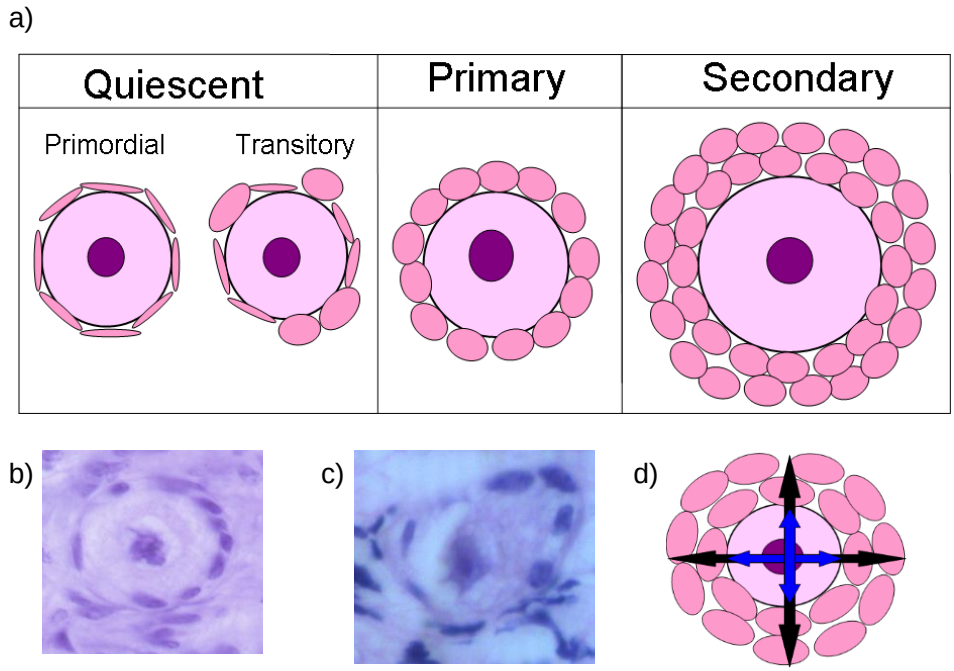


Figure 2.3 Follicle Analysis. a) Classification of ovarian follicles is based on their morphology as quiescent, primary and secondary follicles. The health of the ovarian follicles was also based on morphology and b) represents an example of a healthy follicle and c) represents a follicle that would be classified as unhealthy. Measurements of follicle diameter and oocyte diameter were taken from each follicle d) 2 measurements of the follicle diameter were taken; the first at the largest diameter of the follicle and the second at 90° to the first. The oocyte was measured twice along the same two lines.

2.9 Statistical Analysis

Ovarian fragments and ovarian follicles were analysed to gain better understanding of up regulation of the PI3K pathway, using bpV (HOpic) or 740 Y-P, on primordial follicle activation and subsequent development. This thesis also explored the distribution of the non-growing and growing follicle populations and their viability within the different regions of the ovary. It also compared the activation and development of the ovarian follicles of the bovine and human in an *in vitro* culture environment. The ovarian follicle populations were commonly assessed in this thesis by comparing the concentration of ovarian follicles in the tissue, the distribution of the different developmental stages of the ovarian follicles, the health

of the ovarian follicles as well as assessing the size of the ovarian follicles within each developmental stage.

Analysis of the concentration of ovairan follicles and concetration of healthy quiescent follicles in each region of the ovary

Analysis of the concentration of ovarian follicles and concentration of healthy quiescent follicles in each regions of the ovary by using Kolmogorov Smirnoff test to test for normal distribution of the data. The data were seen to be normally distributed therefore, one-way ANOVA was used to statistically compare the data, where this test reported a significant difference the groups were compared using the post hoc Tukey test. The distribution and health of the ovarian follicles were compared using chi-squared test. Only p-values < 0.05 were considered to be significant.

Chapter Three:

Exploring the Ovarian Medulla as a Potential Source of Primordial Follicles

3.1 Introduction:

The experiments in this thesis were performed with the aim of gaining a better understanding of the quiescent follicle population therefore, knowing their location in the ovary is important. The morphology of the ovary varies widely between mammalian species, however in most species it is possible to identify three main components; the surface epithelium, the cortex and the medulla (Escobar et al., 2011) (see figure 3.1). One fundamental morphological difference in the structure of the ovary between species is the proportion of the cortex and medulla. Some species have very little medulla in their ovaries, such as the mouse, whereas in other species, such as the human, there is a large medullary region in the centre of the ovary (Jimenez, 2010). Structurally the cortex and the medulla are morphologically distinct from one another. The cortex consists of tightly compact connective tissue and is poorly vascularised. In contrast, the medulla comprises loosely connected connective tissue and is highly vascularised (Escobar et al., 2011).

In both the bovine and human the distribution of ovarian follicles within the ovary is known to be different in each region of the ovary, with around 90% of all ovarian follicles located in the cortex. The highest concentration of ovarian follicles is found in the outer cortex, with the ovarian follicle concentration progressively declining through consecutive inner layers of the ovary. The location of ovarian follicles in the ovary is thought to depend upon what stage of follicular development they are in (see figure 3.1). The quiescent follicle population is located predominately in the outer cortex whereas; the growing follicle population is primarily located in the inner cortex and at the cortex-medulla border. Within the medulla there are very few ovarian follicles and those present are generally considered to be at the later stages of folliculogenesis (Jimenez, 2010; vanWezel and Rodgers, 1996).

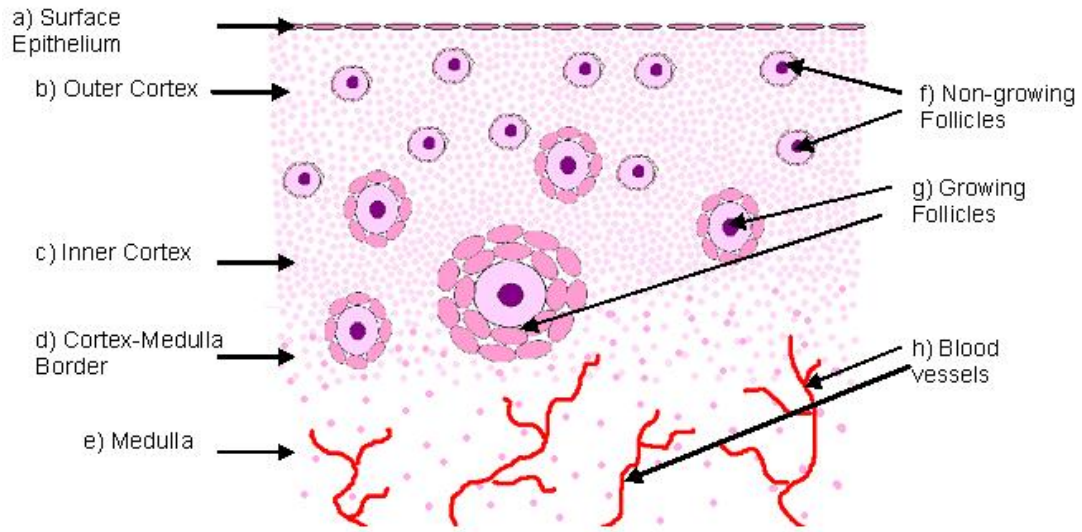


Figure 3.1 Structure of the Ovary and Presence of the Ovarian Follicles within the Different Regions. From the outer edge of the ovary there is the a) surface epithelium, b) outer cortex, c) inner cortex, d) cortex-medulla border and finally the e) medulla. Both the b) outer and c) inner cortex contain tightly connected connective tissue and are poorly vascularised. The (e) medulla has loosely connected connective tissue and is (h) highly vascularised. The (d) cortex-medulla border has characteristics of both the cortex and medulla. (f) Non-growing follicles are found predominately at the (b) outer cortex whereas, the (g) growing follicles are primarily found at the (c) inner cortex and at the (d) cortex-medulla border.

The primordial follicle population is the largest population of ovarian follicles within the ovary at any time (Gosden and Telfer, 1987, vanWezel and Rodgers, 1996). They are also the ovarian follicle population least impacted by cryodamage (Cortvrindt et al., 1997; Hovatta, 2005; Oktay et al., 1997; von Wolff et al., 2009), making them the main target for current and developing fertility preservation techniques. Understanding that the primordial follicle population is primarily in the outer cortex has led to only the cortex to a depth of 1-2mm being preserved from ovarian biopsies as part of current clinical practice for fertility preservation (von Wolff et al., 2009). Additionally, only the cortex is utilised in *in vitro* research examining primordial follicles (Braw-Tal and Yossefi, 1997; McLaughlin and Telfer, 2010; Telfer et al., 2008; Wandji et al., 1996a; Wandji et al., 1997) in species where culturing the whole ovary is not possible due to its large size (Telfer et al., 2000; Thomas et al., 2003; Wandji et al., 1996b). However, a recent study by Kristensen et al., 2011 indicated that there is a population of primordial follicles in the medulla region in the human ovary. Therefore, by only using the

cortex are we losing a potential source of primordial follicles when we discard the medulla?

This chapter will use both bovine and human ovaries that are similar in structure with the same dimensions for the proportion of the cortex and medulla (Jimenez 2010) to examine the ovarian follicle populations within each region of the ovary. The Kristensen et al. 2011 study used neutral red staining to identify ovarian follicles within the medulla. Neutral red is a vital stain and therefore, only binds to viable ovarian follicles. One aim of this chapter was to therefore explore if the health of ovarian follicles was influenced by their location in the ovary, by identifying both the healthy and unhealthy follicles. The Kristensen et al. 2011 study also classified the developmental stage of each ovarian follicles upon follicle size, with follicles <60μm being classified the primordial/early primary. In this study the assessment of the developmental stage of folliculogenesis will be based on morphology, as this will enable a distinction to be made between primordial and early primary (growing) follicles. This should provide a better understanding of the location of the quiescent population and distribution of non-growing and growing ovarian follicles in the ovary.

3.2 Materials and Method

3.2.1 Tissue Collection and Dissection

3.2.1.1 Bovine Samples

Bovine ovaries were collected from the local abattoir and transferred to the laboratory in M199 medium. Each ovary was disinfected with 70% ethanol in a laminar flow hood. Using a surgical blade the superficial 1-2mm of cortex was removed from the surface of 8 different bovine ovaries (see figure 3.2 a). A sample of the ovarian region under this part of the cortex was then removed to a depth of approximately 5-8mm using a surgical blade. The tissue samples (n=8) were then fixed in paraformaldehyde for 48hr, processed, mounted and H&E stained as described in chapter 2.

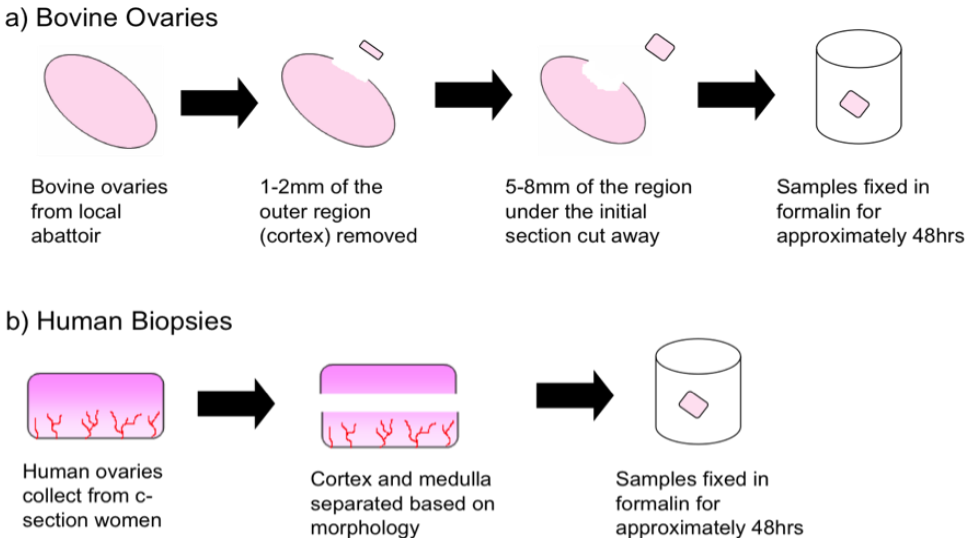


Figure 3.2 Outline of Method. a) Bovine ovaries were collected from the local abattoir. The outer cortex at the surface of the ovary was removed to a depth of 1-2mm from the surface of the ovary. At the section where the outer cortex had been removed another section of tissue was extracted to a depth of approximately 5-8mm. b) Human biopsies were collected from women undergoing elective Caesarean sections. The cortex and medulla were separated based on their morphology. All samples were then fixed in formalin for 48 hours.

3.2.1.2 Human Samples

Human biopsies were collected from 8 different women, aged between 28-38, after informed consent from the local hospitals and transported to the laboratory in human Leibovitz medium. Each biopsy was placed into fresh human Leibovitz medium on arrival for dissection under a laminar flow hood. The more compact cortex (usually 1-2mm in depth) was separated from the vascular medulla (usually 2-5mm in depth) using a surgical blade (see figure 3.2 b). The biopsy samples (n=8) were then fixed in paraformaldehyde for 48 hours, processed, mounted and H&E stained for analysis; for full description see chapter 2.

3.2.2 Analysis

Follicles in H&E stained samples of the human and bovine ovaries were analysed based on their morphology. Ovarian follicles were classified either as quiescent follicles, which included both primordial and transitory follicles or as growing follicles that included both the primary and secondary follicles. Ovarian follicles were also noted to be either healthy or unhealthy based on their morphology using criteria described in chapter 2.

The location of each follicle was determined, and classified as being in the cortex, at the cortex-medulla border or in the medulla based on the morphology of the ovarian tissue surrounding the follicle (see figure 3.3). The cortex was classified as consisting of tightly compacted stromal cells and was non-vascular. The medulla was classified as consisting of less densely compact connective tissue containing blood and lymphatic vessels and nerves, used to support the other regions of the ovary, thereby defined as being highly vascularised tissue. Often there appeared to be no distinction between the medulla and cortex as there were regions in the tissue that had characteristics of both cortex and medulla; these were classified as the cortex-medulla border. Within the bovine tissue the cortex was split into the outer cortex and the inner cortex. The outer cortex consisted of the 1-2mm of the ovarian tissue taken initially from the surface of the ovary whereas, the inner cortex consisted of any cortex found in the 5-8mm sample taken after the initial 1-2mm of the surface of the ovary had been removed. Using Image J 1.44g (Rasband W.S, National Institutes

of Health, USA, http://imagej.nih.gov/ij/) the concentration of ovarian follicles in each region of the ovary was calculated; for a full description see chapter 2.

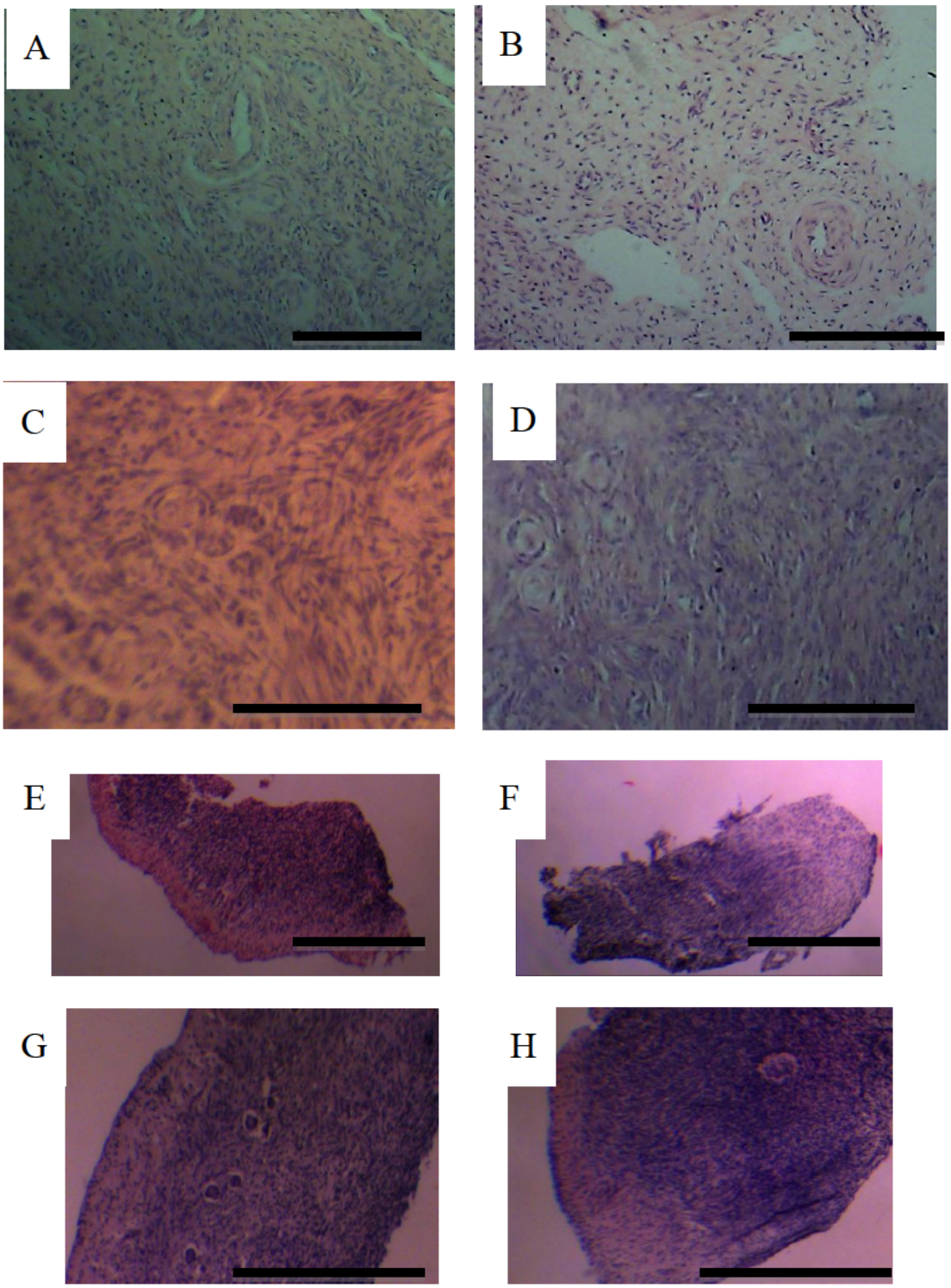


Figure 3.3 Examples of the Morphological Differences Seen Between Each Region of the Ovary from Human and Bovine. Images a) from the bovine and b) from the human display the medulla examples of the medulla consisting of loosely connected connective tissue and containing blood vessels. Images c) from the bovine and d) from the human display the cortex consisting of tightly compact stromal tissue. Images e) and g) from the bovine and f) and h) convey the cortex-medullar border. Scale bar in a and d are equal to 150µm, e and f is equal to 0.5mm and g and h is equal to 0.3mm

3.2.3 Statistical Analysis

Analysis of the concentration of ovarian follicles and concentration of healthy quiescent follicles in each regions of the ovary by using Kolmogorov Smirnoff test to test for normal distribution of the data. The data was normally distributed therefore; a one-way ANOVA was used to statistically compare the data, where this test reported a significant difference the groups were compared individually using a post hoc tukey test. The distribution of the ovarian follicles and health of the ovarian follicles were compared using chi-squared test. Only p-values that were < 0.05 were considered to be significant.

3.3 Results.

3.3.1 Concentration of Ovarian Follicle in Each Region of the Ovary

A significant decrease in the concentration of ovarian follicles was observed between the cortex in the human and outer cortex in the bovine to each progressive inner layer of the ovary. In the human ovarian samples a mean of 17.4±2.3 ovarian follicles/mm^3 were found in the cortex and 5.6±1.5 ovarian follicles/mm^3 in the cortex-medulla. No ovarian follicles were found in the medulla ($p<0.01$) (see figure 3.4 A). In the bovine there were 169.0±12.6 ovarian follicles/mm^3 in the outer cortex, 76.9± 7.1 ovarian follicles/mm^3 in the inner cortex, 32.3±5.0 ovarian follicles/mm^3 in the cortex-medulla and 3.9±0.7 ovarian follicles/mm^3 in the medulla ($p<0.001$) (see figure 3.4 B).

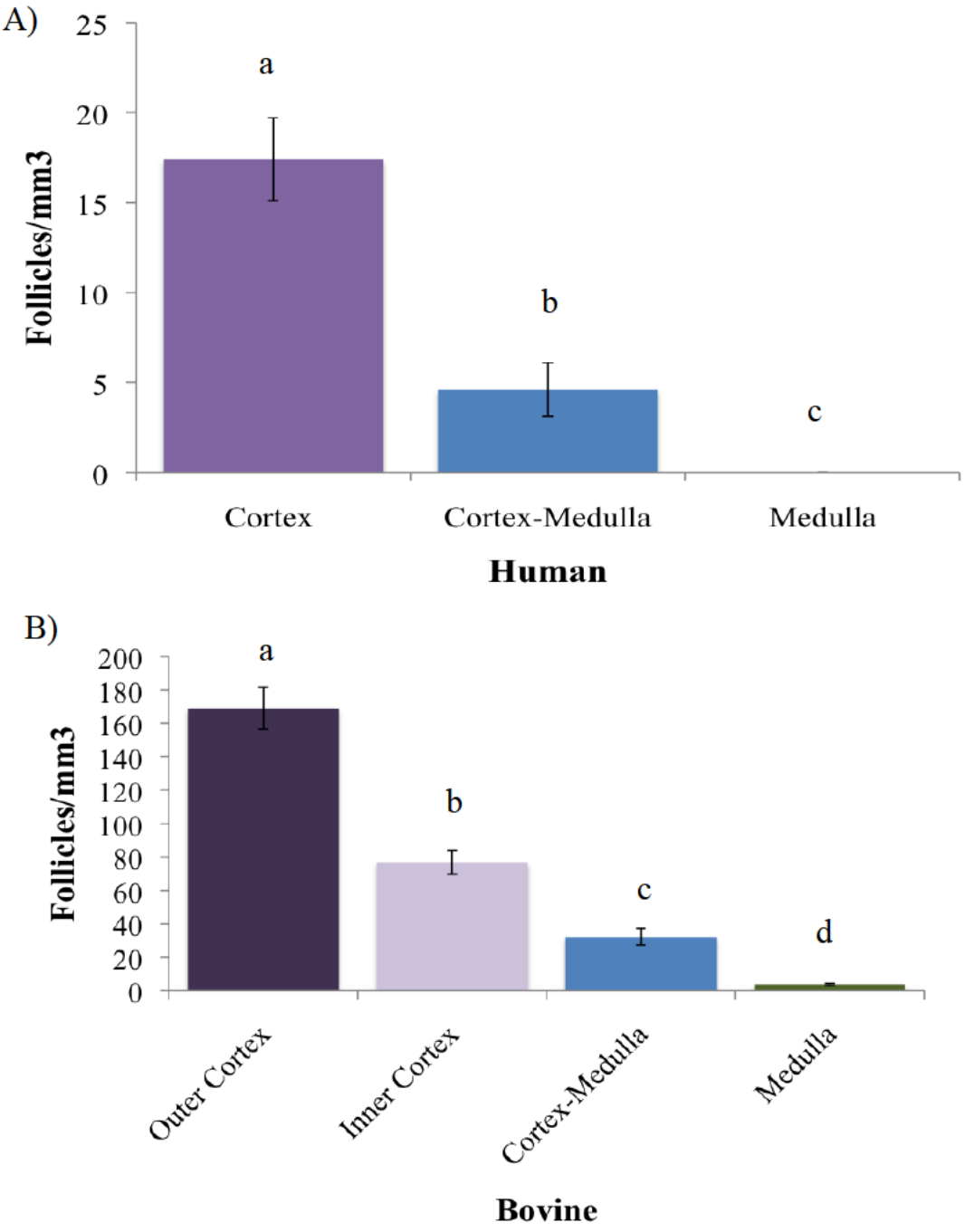


Figure 3.4 Concentration of Ovarian Follicles in Each Region of the Ovary. These graphs display the mean number of ovarian follicles found in $1mm^3$ of each region of the ovary in the a) human ($p<0.01$) and b) bovine ($p<0.001$). Means that have different letters are significantly different from one another ($p<0.01$). In both groups there is a significant decrease the in the concentration of ovarian follicles from the outer region of the ovary through the progressive inner regions of the ovary. Mean±sem, n=8, human; n=8 bovine.

3.3.2 Distribution of Ovarian Follicles

The distribution of the quiescent and growing follicles was calculated in both bovine and human to explore if there were any difference in the distribution queiscent follicles and growing follicles in the different regions of the ovary. Table 3.1 displays a summary of the data collected.

Ovarian follicles were only observed in the cortex and cortex-medulla border in human samples so the distribution of the growing follicles could only be examined in these regions. In the cortex 68.6±2.9% of the ovarian follicles population was quiescent and 31.4±2.9% was growing (see figure 3.5 A). This was significantly different from the cortex medulla where 26.0±2.9% of the ovarian follicle population was quiescent and 74.0±2.9% were growing ($p<0.001$).

In the bovine ovarian tissue samples ovarian follicles were found in all of the regions of the ovary. There was a significant difference in the distribution quiescent and growing follicles in each of the region of the ovary with statistically less quiescent and more growing follicles in the progressively deeper regions of the ovary (see figure 3.5 B). The outer cortex contained 61.9±2.5% quiescent follicles and 38.1±2.5% growing. The inner cortex contained significantly less quiescent follicles and significantly more growing follicles that the cortex with 43.3±1.6% were quiescent with 56.7±1.6% growing ($p<0.001$). The cortex-medullary border contained significantly less quiescent follicles and significantly more growing follicles that the inner cortex at 23.6±2.3% quiescent follicles and 76.4±2.3% growing ($p<0.001$). The medulla contained a low proportion of quiescent ovarian follicles at 8.7±2.4% and a high proportion of growing follicles at 91.3±2.4% than the cortex-medullary border ($p<0.01$).

Between the bovine and human there appears to be a similar increase in the proportion of ovarian follicles that were growing from the cortex to the cortex-medulla border. A 31.5±3.5% and 32.6±3.5% increase in the proportion of the ovarian follicles that were growing follicles in the cortex-medulla border compared to the cortex was observed in both the human and the bovine respectively ($p>0.05$).

Table 3.1: Data Collected from Human and Bovine Samples used to Calculate Distribution

	No. Samples	Region of the Ovary	No. Follicles	No. Quiescent Follicles	No. Growing Follicles
Human	8	Cortex	169	113	56
		Cortex-Medulla	110	26	84
		Medulla	0	0	0
Bovine	8	Outer Cortex	306	190	116
		Inner Cortex	214	93	121
		Cortex-Medulla	170	42	128
		Medulla	65	6	59

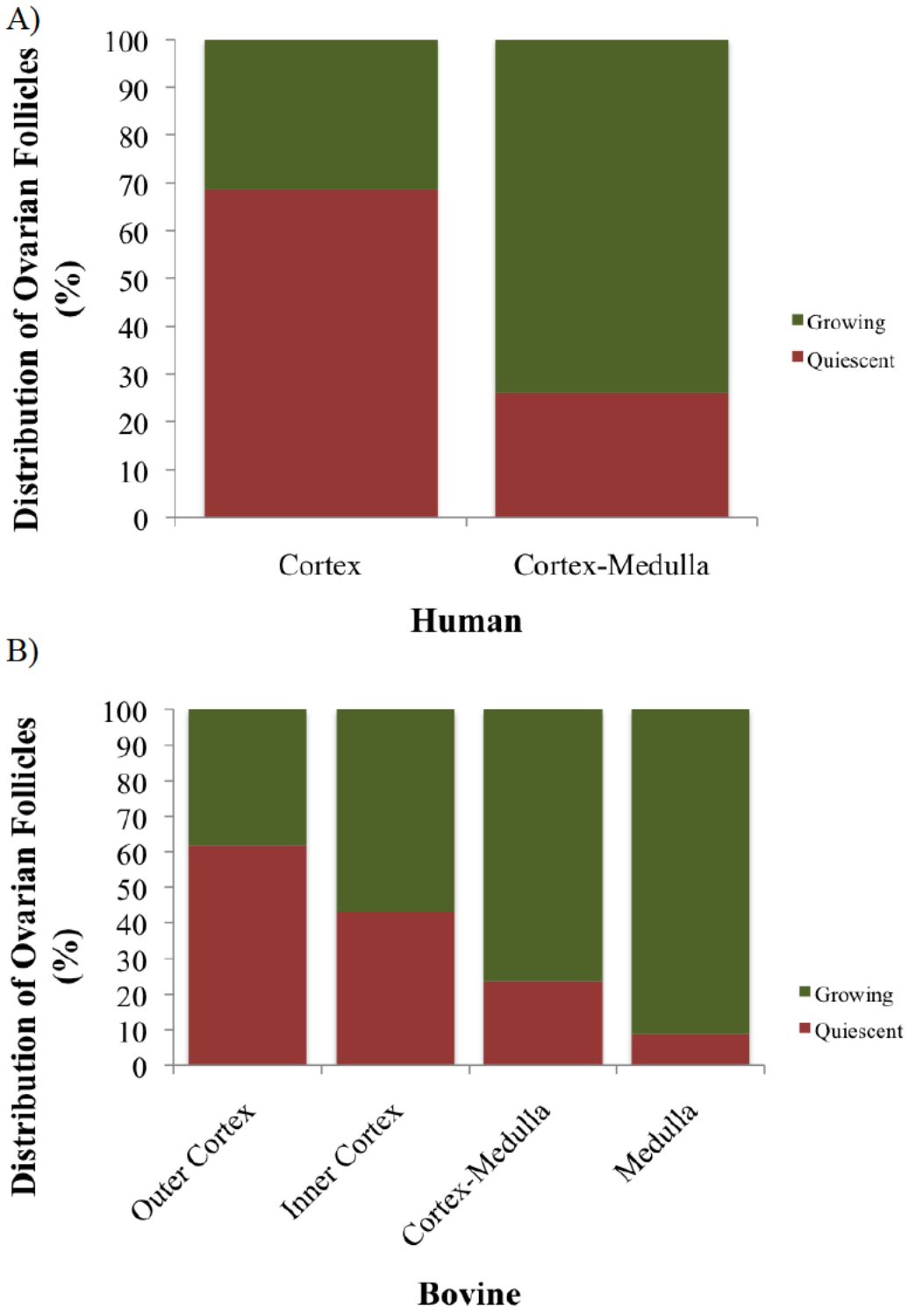


Figure 3.5 Distribution of Ovarian Follicles in Each Region of the Ovary. These graphs display the difference in the distribution of the quiescent and growing follicle populations in the each region of the ovary in the A) human ($p<0.001$) and B) bovine ($p<0.01$). In both species there is a decrease in the percentage of quiescent follicles and increase in the percentage of growing follicles from the outer region of the ovary to the progressive inner regions of the ovary, n=8, human; n=8 bovine.

3.3.4 Health of the Ovarian Follicles

The health of the quiescent and growing follicles was calculated in both bovine and human for each region of the ovary to examine to if the location had any impact on the health of the follicles. Table 3.2 displays a summary of the data collected.

The health of ovarian follicles was influence by their location in the ovary in both bovine and human samples. The proportion of healthy quiescent follicles was seen to decrease significantly from 65.3±2.5% in the cortex to only 47.1±6.0% in the cortex-medulla border in the human ($p<0.05$) (see figure 3.6). There was also a significant decrease in the health of the growing follicles in the cortex at 58.3±4.8% to only 39.9±2.5% in the cortex-medulla border ($p<0.05$).

In the bovine ovary there was no significant difference in the health of the quiescent follicles in the outer cortex at 71.3±3.3% and the inner cortex at 70.5±.3.1% ($p>0.05$) (see figure 3.7 and 3.8). However, there was a significant decrease in the health of follicles in the cortex-medulla at only 28.8±4.6% compared to the cortex ($p<0.001$). In the medulla only 16.7±14.4% of the quiescent follicle population were seen to be healthy but too few quiescent follicles were found in the medulla region to carry out any statistical tests. The growing follicles displayed no significant difference in health with 66.3±3.6% and 63.5±2.6% of the ovarian follicles being healthy in the outer cortex and inner cortex respectively ($p>0.05$). There was a significant decrease in the health of the growing follicles in the cortex-medulla border at 27.6±1.3% compared to the cortex ($p<0.001$). The growing follicles were significant lower in health at only 8.0±2.6% compared to the cortex-medulla border ($p<0.05$).

In order to compare the difference in the health of the ovarian follicles in the different regions of the ovary between the human and the bovine, the outer and inner cortex of the bovine were combined (as there was no significant difference in follicular health) to create a single cortex group to compare to the cortex-medulla. The medulla could not be compared between the two as no ovarian follicles were observed in the medulla of the human. The decrease in health of the ovarian follicles from the cortex to the cortex-medulla border was seen to be similar at 18.1±4.1% and

18.2±3.9% for quiescent and growing follicle populations respectively in the human ($p>0.05$) (see figure 3.9). Similarly, the difference in health of the both the quiescent and growing follicles did not differ between the cortex and cortex-medulla in the bovine at 42.2±2.7% and 37.9±2.6% respectively ($p>0.05$). No comparison could be made between the health of the quiescent and growing follicles population in the cortex-medulla to the medulla in the bovine as there were too few quiescent follicles were found in the medulla region to carry out any statistical tests. However, the difference in the health of the ovarian follicles between the cortex and cortex-medulla was significantly higher in the bovine in comparison to human for both the quiescent and growing follicle populations ($p<0.05$).

Table 3.2: Data Collected from Human and Bovine Samples used to Calculate Follicular Health in the Quiescent and Growing Follicle Populations.

	No. Samples	**Region of the Ovary**	**No. Heathy Quiescent Follicles**	**No. Unheathy Quiescent Follicles**	**No. Heathy Growing Follicles**	**No. Unheathy Growing Follicles**
Human	8	Cortex	74	39	32	27
		Cortex-Medulla	11	14	31	52
		Medulla	0	0	0	0
Bovine	8	Outer Cortex	139	51	77	39
		Inner Cortex	64	29	78	43
		Cortex-Medulla	13	29	36	92
		Medulla	1	5	6	55

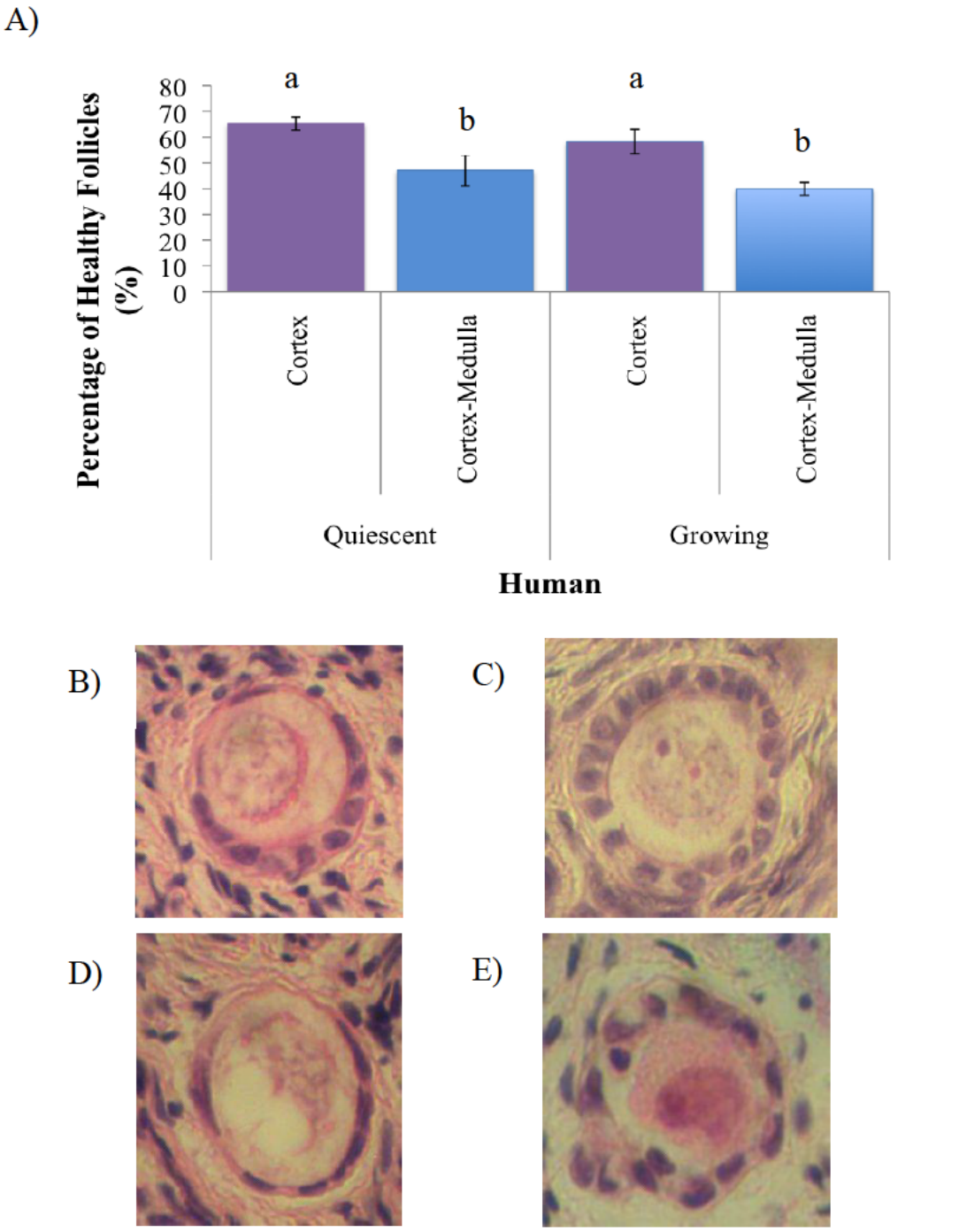


Figure 3.6 Follicular Health of the Quiescent and Growing Follicles in the Different Regions of the Ovary in the Human. A) Displays a graph showing how the decrease in health of both the quiescent and growing follicles in cortex medulla in comparison to the cortex. Means that have different letters are significantly different from one another in quiescent and growing follicle populations ($p<0.05$). Mean±sem, n=8. Example of this change in the health of the quiescent follicles can be seen between B) quiescent and C) growing follicles from the cortex in comparison to the D) quiescent and E) growing follicles from the cortex-medulla.

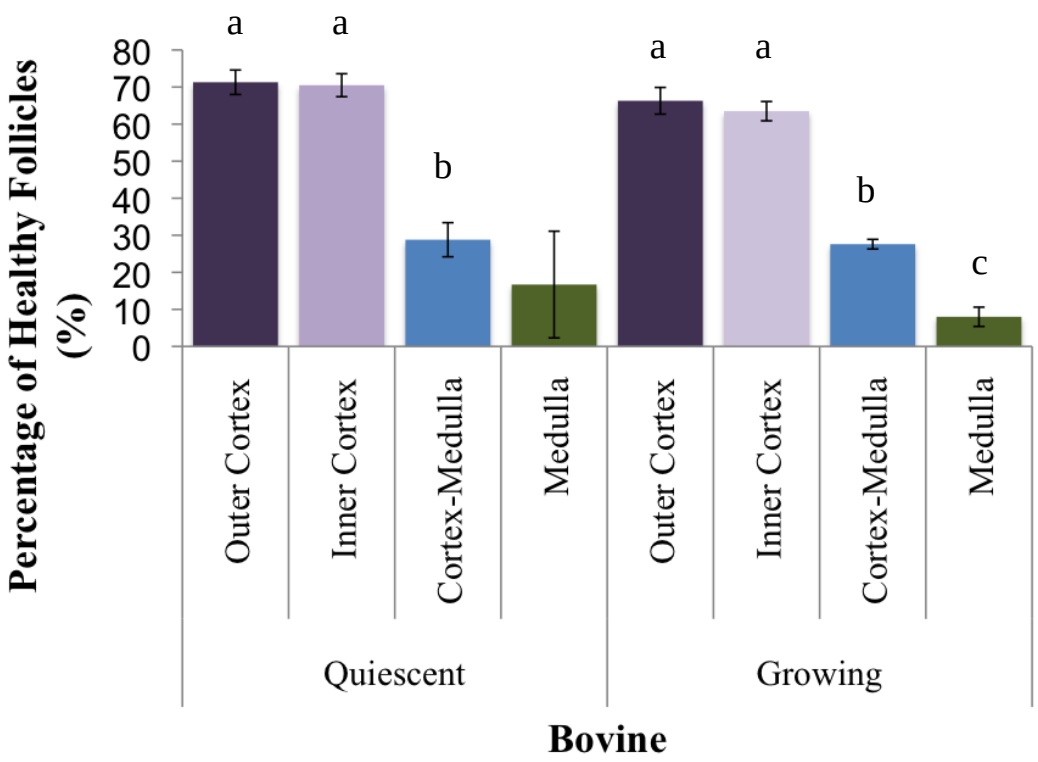
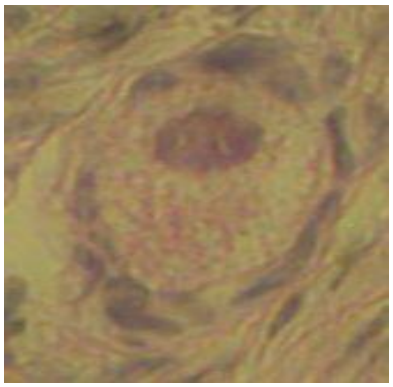
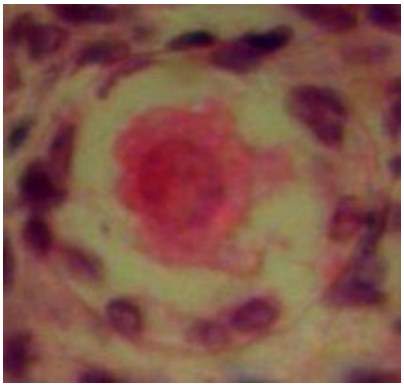


Figure 3.7 Follicular Health of the Quiescent and Growing Follicles in the each Region of the Ovary in the Bovine. Graph displays no change in the health of quiescent follicles in the outer and inner cortex but a decrease in the health is observed in the cortex-medulla. There is a decrease in the health of the quiescent and growing follicles from the outer and inner cortex in comparison to the cortex-medulla and a further decrease in the health of growing follicle population found in the medulla. Means that have different letters are significantly different from one another within quiescent and growing follicle populations ($p<0.05$). Mean±sem, n=8.

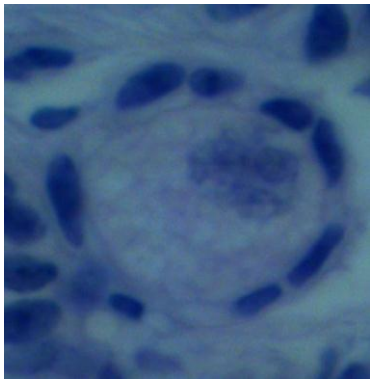
A)



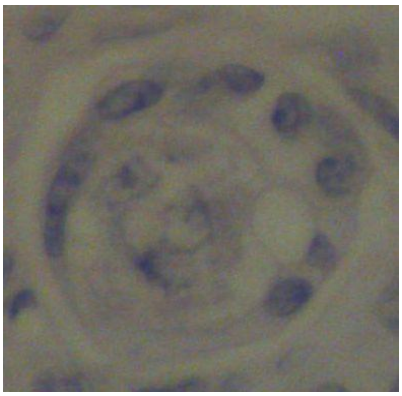
B)



C)



D)



E)

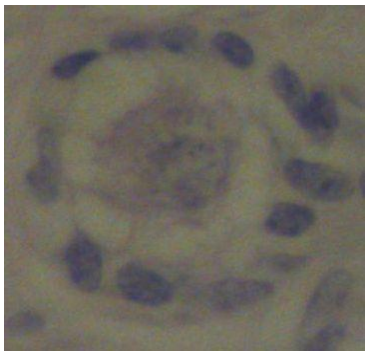


Figure 3.8 Quiescent and Growing Follicle Health in the Different Regions of the Bovine Ovary. Example of the health of quiescent follicles found in A) cortex and B) the cortex-medulla border. There is also an example of a healthy growing follicle from the C) cortex, D) cortex-medulla and E) medulla.

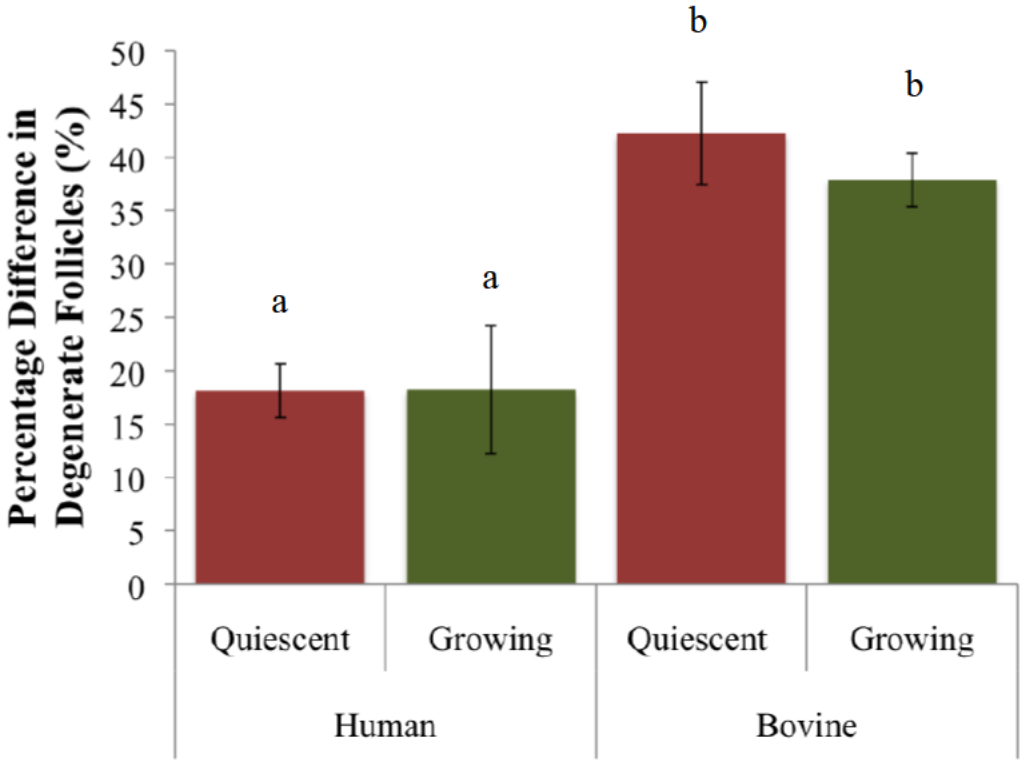


Figure 3.9 Difference in the Percentage of Degenerating Follicles Observed in the Cortex-medulla Border in Comparison to the Cortex.
The percentage increase in proportion of degenerate follicles stays the same between the quiescent and growing follicles in the human and the bovine between the cortex and cortex-medulla border. However, there is a significantly higher proportion of degenerate quiescent and growing follicles in the bovine comparison to the human. Means that have different letters are significantly different from one another ($p<0.05$). Mean±sem, n=8, human; n=8 bovine.

3.3.5 Concentration of Healthy Quiescent Follicles in Each Region of the Ovary

In both human and bovine there was a significant difference in the number of healthy quiescent follicles between the different regions of the ovary. In the human there were significantly more healthy quiescent follicles in the cortex at 9.9±2.5 compared to 1.5±1.1 healthy quiescent follicles/mm^3 in the cortex-medulla and no healthy quiescent follicle in the medulla ($p<0.001$) (see figure 3.10). In the bovine there were 78.8±5.7 healthy quiescent follicles/mm^3 in the outer cortex, 27.8±3.5 healthy quiescent follicles/mm^3 in the inner cortex, 2.8±0.5 /mm^3 in the cortex-medulla and 0.1±0.1 healthy quiescent follicles/mm^3 in the medulla ($p<0.001$).

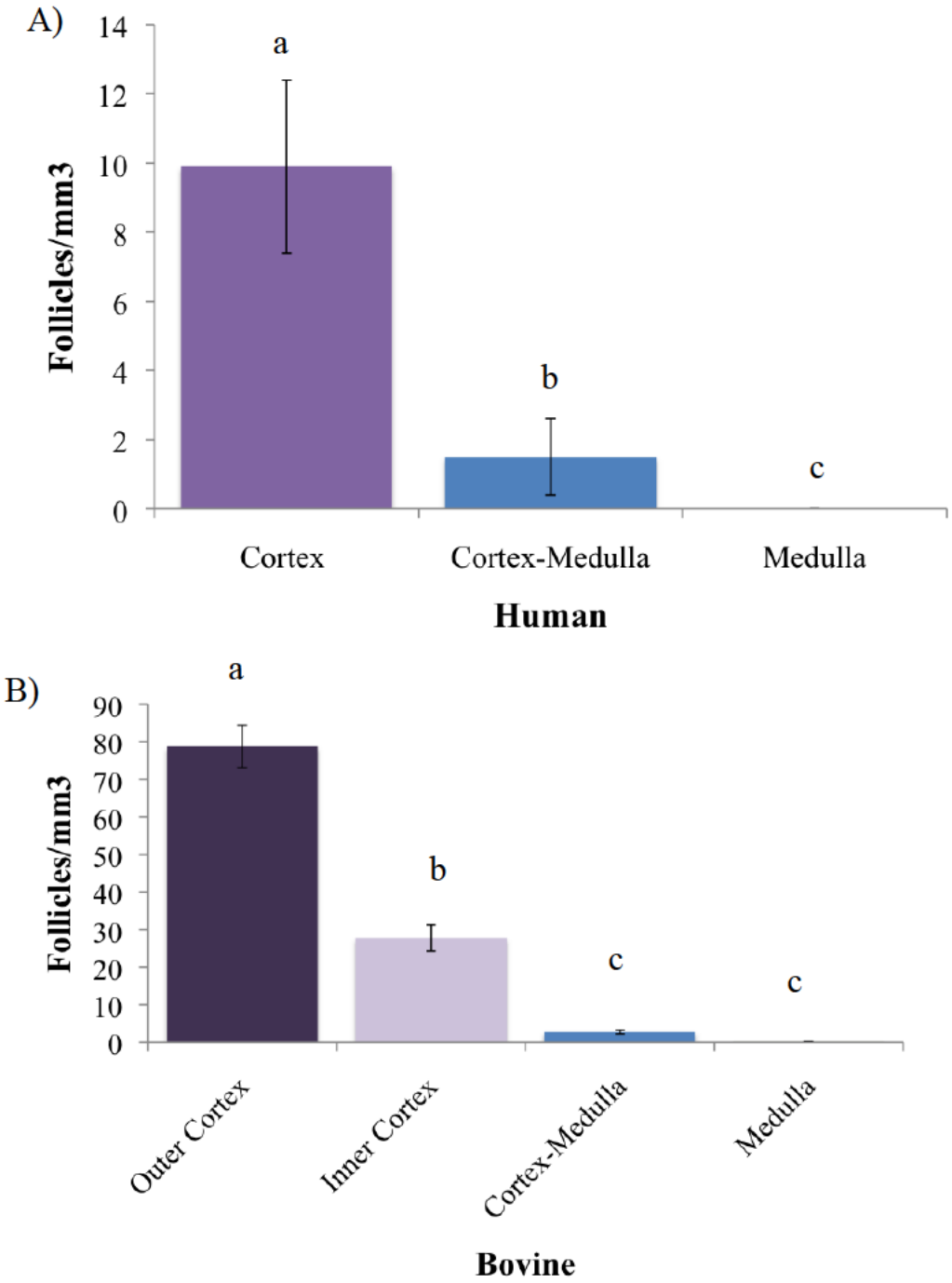


Figure 3.10 Concentration of Healthy Quiescent Follicles in the Different Regions of the Ovary. These graphs display the mean number of healthy quiescent follicles found in $1mm^3$ of each of the different regions of the ovary of the A) human ($p<0.001$) and B) bovine ($p<0.001$). Means that have different letters are significantly different from one another ($p<0.001$). In both animals there is a significant decrease the in the concentration of healthy quiescent follicles from the outer region of the ovary through the progressive inner regions of the ovary. Mean±sem, n=8, human; n=8 bovine.

3.4 Discussion

The aim of these experiments was to examine the medulla as a potential source of ovarian follicles and in particular to explore the location of the quiescent follicle population in the ovary. This was done in both bovine and human, which have ovaries that are similar in structure with the same dimensions for the proportion of the cortex and medulla (Jimenez 2010). The number of ovarian follicles/mm^3 was seen to decrease in both bovine and human from outer cortex and cortex respectively and through the progressive inner layers of the ovary. This agrees with the change in concentration of the ovarian follicles from the outer region to the inner regions of the ovary previously observed in the bovine and human (Jimenez, 2010; vanWezel and Rodgers, 1996). In this study the growing follicles population consisted of predominately primary follicles as well as secondary follicles.

In human no ovarian follicles were observed in the portion of the medulla obtained from each biopsy. This differs from the previous study by Kristensen et al., 2010, where they found ovarian follicles in the medulla at all ages. However, they classify ovarian tissue as either cortex or medulla based on distance from the outer edge of the ovary. Based on the cortex usually spanning 1-2mm (Kristensen et al., 2011; von Wolff et al., 2009) the first 1-2mm was classified as cortex and the rest as medulla. Within this study the morphology of the tissue based on H&E staining was used to identify not only the cortex and medulla but also the cortex-medulla border on the basis of structure rather than distance from the edge of the ovary (Escobar et al., 2011; vanWezel and Rodgers, 1996). It is likely that the cortex-medulla region found in this study was included in the medulla region in Kristensen et al., 2011, and that these different methods of classifying the regions of the ovary account for the differences. Another factor could be the size of the human biopsies received in this study compared to the Kristensen et al., 2011 study, as only a small biopsy of the ovary was taking from the caesarean-section patients within this study whereas, in the Kristensen et al., 2011 they had much large tissue samples as they received the medulla from a whole ovary. Another factor to considered is the range of ages used within this study both the human and the bovine ovarian samples range from 28-38 years and 10-14 months in age respectively and therefore, only taken at a post-pubertal age whereas, the study Kristensen et al., 2011 were collected from a much

wider age range from 3 to 35 years. In the Kristensen et al. 2011 study it was shown that the presence of ovarian follicles within the medulla was much more prevalent in the pre-pubertal ages which was not explored within this study.

The decrease in number of ovarian follicles from the outer edge of the ovary to the inner regions of the ovary could be due to a number of reasons. It was found that the proportion of quiescent ovarian follicles decreased from the cortex in the human ovaries and the outer cortex in the bovine ovaries through the progressive deeper layers of the ovary. Subsequently, the majority of ovarian follicles in the cortex-medulla and medulla were growing follicles, again agreeing with that observed in previous studies (vanWezel and Rodgers, 1996). The structure of the ovarian follicles and the timing of their formation are thought to influence the activation of the ovarian follicles. It is hypothesized that ovarian follicles follow the production line hypothesis proposed by Henderson and Edwards in 1968 which states that those primordial follicles that are the first to enter meiosis are the first to undergo folliculogenesis (Henderson and Edwards, 1968). The primordial follicles in the cortex are the last to be made resulting in it having the largest portion of quiescent follicles (Hirshfield, 1992; Mork et al. 2012).

A study on the mouse ovary found that the first primordial follicles are formed within the medulla region (Mork et al. 2012). Cells known as progenitors are found within the cortex are the cells that give rise to the cortical granulosa cells as they proliferate. This timing of formation of the ovarian follicles means that the ovarian granulosa cells within these ovarian follicles that are formed earlier and are created from the progenitor at an earlier stage in its development (Mork et al., 2012). The ovarian follicles with granulosa cells formed in the progenitor's early development may be unable to maintain the dormancy of the quiescent follicles as they are proposed to be missing a factor that is acquired at a later stage of the progenitor's development. The follicles that have these granulosa cells are predominately found in the medulla region of the ovary (Mork et al., 2012), as a result the primordial follicles in the medulla are part of the first wave of primordial follicles activated (Hirshfield 1992, Eppig and Handel 2012). Although this has only been recorded in the mouse model it is possible that it occurs in other species; the change in the distribution quiescent and growing follicles in both the bovine and

human showing a lower proportion of quiescent in the inner regions of the ovary could indicate that this also occurs these two species. Once an ovarian follicle has undergone activation it will either continue though the various stages of folliculogenesis until it reaches ovulation or it will undergo atresia (Scaramuzzi et al., 2011; Webb and Campbell, 2007; Webb et al., 2004). This could account for the decrease in the number of ovarian follicles and decrease in the percentage of the ovarian follicles that are quiescent in the inner regions of the ovary in comparison to the outer regions of the ovary.

Growing follicles are thought to move away from the outer cortex towards the medulla. The change in the physical environment is thought to influence the growth process of the ovarian follicles. It has been hypothesised that the more rigid ovarian cortex is relatively non-permissive for the growth of ovarian follicles whereas the perimedullar zone is thought to be relatively permissive for follicle growth. Within this hypothesis it is thought that the biomechanical environment in the different zones of the ovary are able to modulate the follicles response to hormones. The ovarian follicles are thought to be able to sense a dynamic environment in the different zones as they grow to expand outwards and move through the stroma into the more permissive environment for growth (Woodruff and Shea, 2011). In this study there was a difference in the density of stromal cells in the different regions of the ovary, which became progressively less dense from the cortex to the inner regions of the ovary. The movement of the growing ovarian follicles from the dense stromal region of the outer ovary to the less dense regions of the inner region of the ovary might be more permissive for follicular growth, and could be another factor contributing to the change in distribution of quiescent and primary follicles in each region of both the bovine and human ovaries observed in this study.

There is also a decrease in the health of both quiescent and growing follicle populations from the cortex and cortex-medulla, and a further decrease in the health of growing follicles found in the medulla of the bovine. The health in both the growing and quiescent follicle population showed a similar decrease in the cortex-medulla compared to the cortex in both species. This indicates that factors impacting the health of the ovarian follicles impact both the quiescent and growing follicles

populations equally. However, no significant difference was observed in the health of the ovarian follicles in the outer and inner cortex of the bovine tissue, indicating that the different environments present within the different regions of the ovary could influence the health of the ovarian follicles. How ovarian environment might influence the process of folliculogenesis is unclear. One factor could be the disruption of the interaction of the granulosa cells with the surrounding stromal cells of the cortex to induce differentiation of the theca cells, which does not occur with medullary stromal cells (Orisaka et al., 2006). The decrease in health could be a correlation with the reduction of cortical stromal cells within the cortex-medulla and their absence in the medulla itself accounting for a progressive increase in degenerate follicles from the cortex to the cortex-medulla border and finally the medulla. The higher proportion of degenerate ovarian follicles in the inner regions of the ovary could also be attributed to the decrease in the number of ovarian follicles from the cortex to the cortex-medulla and medulla as the quality of the ovarian follicles is known to be a key factor that causes an ovarian follicle to undergo atresia (Blondin and Sirard, 1995; Hendriksen et al., 2004; Salamone et al., 1999).

Although there is a decrease in the proportion of healthy ovarian follicles in the medulla and cortex-medulla, a recent study by Wilken-Jensen et al 2013 has indicated that it is still possible for a mature oocyte to be extracted from biopsy material left after the cortex has been removed, using human ovarian tissue. Currently, in many cases the aim of cryopreserving ovarian tissue is for it to be used later to help the individual have a baby. Therefore, from a clinical aspect being able to utilise these ovarian follicles from all the regions of the ovary is important (Wilken-Jensen et al., 2013).

In summary, there is a decrease in the number of ovarian follicles from outer cortex to the inner regions of the ovary, in both human and bovine ovaries. The location of the ovarian follicle population impacts both the distribution of quiescent and growing follicles and the health of the ovarian follicles. The ovarian follicles in the outer cortex observed to be predominantly quiescent, the proportion of quiescent follicles is seen to decline through the progressive inner regions of the ovary. The health of ovarian follicles was also reduced from the cortex to the inner regions of the ovary in both the quiescent and growing follicle population, indicating the

environment of the inner regions of the ovary influences the health of the follicles. As a result there are very few healthy quiescent follicles in the inner regions of the ovary compared to the cortex, and indeed none were found in the medulla in the human biopsies studied. Therefore, when exploring the quiescent follicle population in later chapters in this thesis only the cortex will be utilised.

Chapter Four:
Exploring the Bovine as a Model for Human Primordial Follicle Activation.

4.1 Introduction

Studies exploring the role of the PI3K pathway on primordial follicle fate have been predominately based in the rodent model. These models have given a good indication of the importance of the PI3K pathway in both primordial follicle activation (Castrillon et al., 2003; John et al., 2008; Liu et al., 2007; Rajareddy et al., 2007; Reddy et al., 2008) and dormancy (Liu et al., 2007; Reddy et al., 2009). However, it is important to explore if the PI3K pathway plays the same role in other species. Rodents are small poly-ovulatory mammals (Milligan et al., 1980) therefore, this thesis aimed to explore if the PI3K pathway plays the same role in a large mono-ovulatory species. The chosen animal model is a large mono-ovulatory species with similar attributes in its reproduction and folliculogenesis to the human (Gougeon, 1986), as this will not only allow the experiments in this thesis to show the role of the PI3K pathway in another species, but also give a valuable indication to its role in the human.

Domestic species have been highlighted to be good models for human as the ovarian follicle size at each developmental stage and the rate at which the ovarian follicles grow is similar (Baerwald, 2009; Gougeon, 1996). In particular, the bovine has been indicated to be a good model because they share many characteristics to humans in both their reproduction and folliculogenesis. Both the bovine and the human are mono-ovulate and undergo estrous/menstrual cycles throughout the entire year (Adams and Pierson, 1995; Baerwald, 2009), in contrast to other domestic animals which are seasonal breeders, such as sheep (Rosa and Bryant, 2003), or polyovulatory, such as the pig (Cortvrindt and Smitz, 2001a; Dziuk, 1977). Bovine ovaries are also close in size to the human, with both being approximately 3cm by 2cm by 1.5cm in size (Adams and Pierson, 1995). The structures observed in bovine and human ovaries are also similar and have proportional amounts of cortex and medulla (Jimenez, 2010).

The pool of primordial follicles is established before birth in both species. In the bovine there are approximately 133,000 primordial follicles at birth. This number remains stable until about the 4^{th}-6^{th} year of life after which the number of primordial follicles declines to approximately 25,000 at 10-14 years of age and by 15-20 years

of age there are only around 3,000 primordial follicles remaining. The bovine reaches reproductive cessation at around 25 years (Erickson, 1966; Erickson, 1976; von Saal et al., 1994; Cohen, 2004). At birth in the human there are approximately 600,000-700,000 primordial follicles. Studies have suggested the number primordial follicle declines steadily until around 37 years of age where there are approximately 25,000 follicles remaining. After 37 years there is thought to be an increased rate of decline in the number of primordial follicles until eventually menopause is reached at 50-51 years (Block, 1953; Gougeon and Chainey, 1987, Richardson et al., 1987, Forabosco et al., 1991; Faddy et al., 1992; Faddy and Gosden, 1996).

Ovarian follicular dynamics and endocrine control in the bovine and human also resemble one another (Adams and Pierson, 1995; Baerwald et al., 2003). *In vivo* development of the primordial follicles to become pre-ovulatory follicles is seen to take similar amount of time in the human and the bovine (Gougeon, 1986; Lussier et al., 1987; Russe, 1983). The sizes of the ovarian follicles at each developmental stage are also within a similar range in both (Braw-Tal and Yossefi, 1997; Gougeon, 1996) (see figure 4.1), which indicates that there is a similar amount and rate of growth occurring in the ovarian follicles of both species as the ovarian follicles progress through the various stages of folliculogenesis. The bovine and the human have similar pathological conditions such as follicular cysts, luteinized anovulatory follicles, lactation- and stress- related suppression of follicle growth and ovulation (Adams and Pierson, 1995) and decreased fertility with maternal aging (Erickson et al., 1976; Klein and Sauer, 2001). These similarities not only in the reproduction and folliculogenesis of the bovine and human but also in pathological conditions indicate that the bovine would be a good model for the human. The aim of this chapter was therefore, to explore if the bovine was a good model for human primordial follicle activation *in vitro*, which will be achieved by using the *in vitro* two-step culture technique developed by McLaughlin and Telfer, 2010 and Telfer et al., 2008. This chapter will compare proportion of quiescent follicles activated and the subsequent growth of the ovarian follicles between the two species *in vitro*.

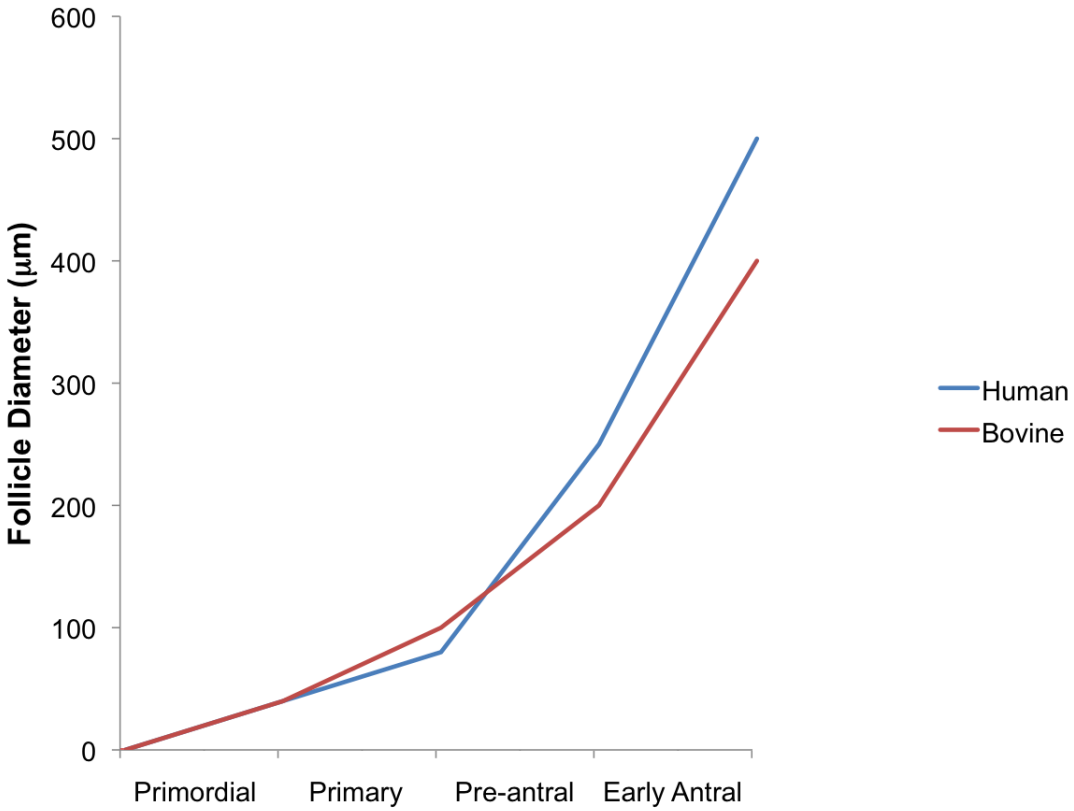


Figure 4.1 A Comparison of Ovarian Follicle Diameters at the Different Developmental Stages in the Bovine and Human during Early Folliculogenesis. This graph displays the increase in the diameters of the ovarian follicles in the bovine and the human through the progressive stages of folliculogenesis from the primordial stage to the early antral stage. At each stage of development the diameters of the ovarian follicles in the bovine and the human are similar (data from Gougeon, 1996 and Braw-Tal and Yossefi, 1997).

4.2 Materials and Methods

4.2.1 Tissue Collection, Dissection and Culture

Bovine ovaries were collected from the local abattoir from animals aged between 10-14 months (n=12) and transported to the laboratory in supplemented M199 medium. Human biopsies were collected from local hospitals after informed consent from women undergoing an elective caesarean section (n=10), and transported to the laboratory in human Leibovitz medium; for a full description see chapter 2. The cortex was extracted from 12 different bovine ovaries and human samples from 10 different women (see figure 4.2), and examined under a light microscope in bovine Leibovitz medium or human Leibovitz medium ensuring that all the ovarian follicles within the cortical strips were less than 40µm in diameter. These strips of cortex were then dissected to approximately 0.5-1 mm^2 in size. Both the bovine and the human samples were cultured with bovine McCoy's or human McCoy's medium respectively for 6 days in a humidified incubator at 37°C and 5% CO_2. Every 48hr the medium of both was changed by removing 150µl of the current medium and replacing it with 150µl of fresh control medium. After 6 days in culture the ovarian tissue was fixed, processed and H&E stained, for a full description see chapter 2.

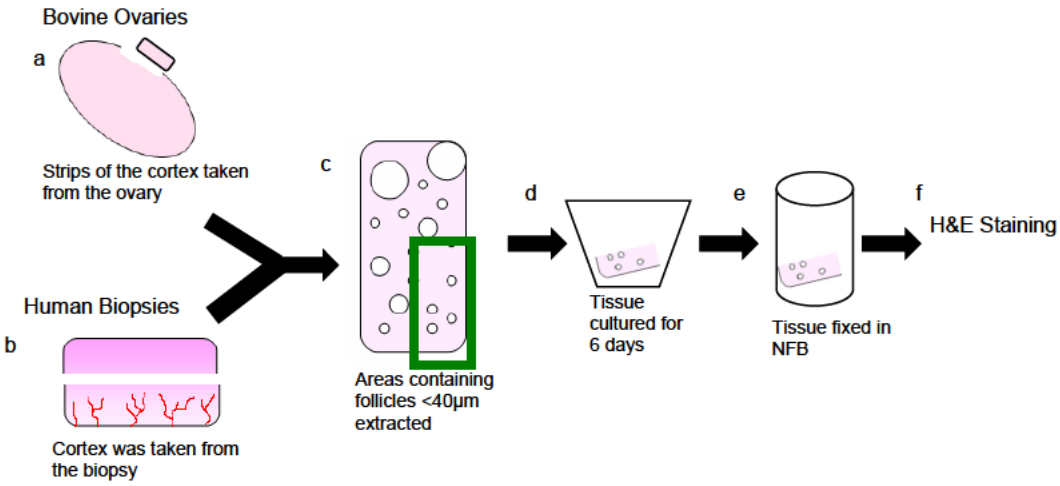


Figure 4.2 Outline of Method. a) Strips of the cortex are taken from the bovine ovaries and b) the cortex is extracted from the human biopsies. c) Both the bovine and human cortex was examined and only areas with follicles of <40µm in diameter were kept. d) The cortical fragments for both the bovine and human tissue was cultured for 6 days in bovine or human McCoy's medium respectively e) after 6 days the tissue was fixed in formalin, processed and then f) H&E stained.

4.2.2 Analysis

Ovarian cortical samples from both bovine and human were H&E stained for morphological analysis. The morphology of the ovarian follicles allowed for each of them to be classified as either quiescent, which included both primordial and transitory follicles, or as primary or secondary follicles. Ovarian follicles were also measured using light microscopy with an eyepiece graticule. Each ovarian follicle was classified as healthy or unhealthy, again based on morphology. The ovarian follicle concentration was calculated using ImageJ 1.44g (Rasband W.S, National Institutes of Health, USA, http://imagej.nih.gov/ij/). Full parameters for classification of the ovarian follicles developmental stage, health and calculation of concentration can be seen in chapter 2.

4.2.3 Statistical Analysis

The distribution of the ovarian follicles between day 0 and day 6 was compared in both species and between the bovine and the human at day 0 and day 6

using a chi-square test. The change in the distribution of the ovarian follicle populations and the health of the different ovarian follicle populations between day 0 and day 6 in bovine and the human repectively were compared using a chi-square test.

Levels of activation, change in the ovarian follicle population, diameters of the quiescent follicles and primary follicles, the increase in the diameter of the ovarian follicles from quiesent to primary and the concentration of the ovarian follicles within the samples were tested for normal distribution using Kolmogorov Smirnoff test, further statistical tests were chosen based on whether the data was normal distributed or not. The level of activation and the increase in the proportion of ovarian follicles that were part of the primary and secondary follicle populations were calculated from day 0 to day 6 and were compared using a two-sampled t-test for both the bovine and the human. The average diameters of the quiescent and primary follicles in both the bovine and the human on both day 0 and day 6 were compared using a two-sampled t-test. The average increase in the diameter of the ovarian follicles and their oocytes from the quiescent to primary stage of development were compared using a two-sampled t-test within both species. The concentration of ovarian follicles in the bovine and human were compared using a two-sampled t-test. Only p-values that were <0.05 were considered to be significant.

4.3 Results

4.3.1 Culture of the Human and Bovine Tissue *In Vitro*

The activation of the quiescent follicles in the *in vitro* culture system were first explored individually in both human and bovine tissue by examining the changes in follicular distribution. Table 4.1 displays a summary of the data collected between the untreated day 0 samples and the cultured day 6 samples for both species**.**

In both human and bovine there was a significant change in the distribution of the ovarian follicle populations (see figure 4.3). In the human at day 0 the distribution of the ovarian follicle populations showed that 63.6±2.6% comprised the quiescent follicle population, 33.3±1.9% comprised the primary follicle population and 2.8±1.4% comprised the secondary follicle population. After 6 days in culture the distribution of ovarian follicles was seen to change with a significant decrease in the quiescent population to only 36.2±4.4% of the ovarian follicle population ($p<0.001$) and a significant increase in both the primary follicle population to 54.4±2.1% ($p<0.001$) and secondary follicle population to 9.4 ±2.9% ($p<0.05$) of the ovarian follicles population.

In the bovine at day 0 the distribution of ovarian follicles showed that 60.3±3.6% comprised the quiescent follicle population, 36.0 ±3.6% comprised the primary follicle population and 3.7 ±1.1% comprised the secondary follicle population. After 6 days in culture the distribution of the ovarian follicle populations changed with a significant decrease proportion of ovarian follicles in the quiescent follicle population to only 36.4±1.2% ($p<0.001$) of the ovarian follicle population and a significant increase in both the primary follicle population to 55.7 ±1.5% ($p<0.001$) and secondary follicle population to 7.9 ±0.9% ($p<0.001$) of the ovarian follicle population.

The health of the ovarian follicles before and after being cultured *in vitro* were first explored individually in both human and bovine tissue. Table 4.2 displays a summary of the data collected between the untreated day 0 samples and the cultured day 6 samples for both species**.** For both human and bovine there was seen to be no significant difference in the proportion of healthy ovarian follicles in the

quiescent, primary or secondary follicle population between day 0 and day 6 ($p>0.05$) (see figure 4.4).

Table 4.1: Data Collected from Human and Bovine Samples used to Calculate Distribution

	Human		**Bovine**	
	Day 0 Uncultured	Day 6 Control Cultured	Day 0 Uncultured	Day 6 Control Cultured
No. Samples	10	10	12	12
No. Follicles	178	209	353	345
No. Quiescent Follicles	108	75	205	125
No. Primary Follicles	63	112	138	193
No. Secondary Follicles	7	22	10	27

Table 4.2: Data Collected from Human and Bovine Samples used to Calculate Follicular Health

	Human		**Bovine**	
	Day 0 Uncultured	Day 6 Control Cultured	Day 0 Uncultured	Day 6 Control Cultured
No. Samples	10	10	12	12
No. Healthy Quiescent Follicles	81	56	127	82
No. Unhealthy Quiescent Follicles	27	19	78	43
No. Healthy Primary Follicles	35	67	88	124
No. Unhealthy Primary Follicles	28	45	50	69
No. Healthy Secondary Follicles	3	9	4	14
No. Unhealthy Secondary Follicles	4	13	6	13

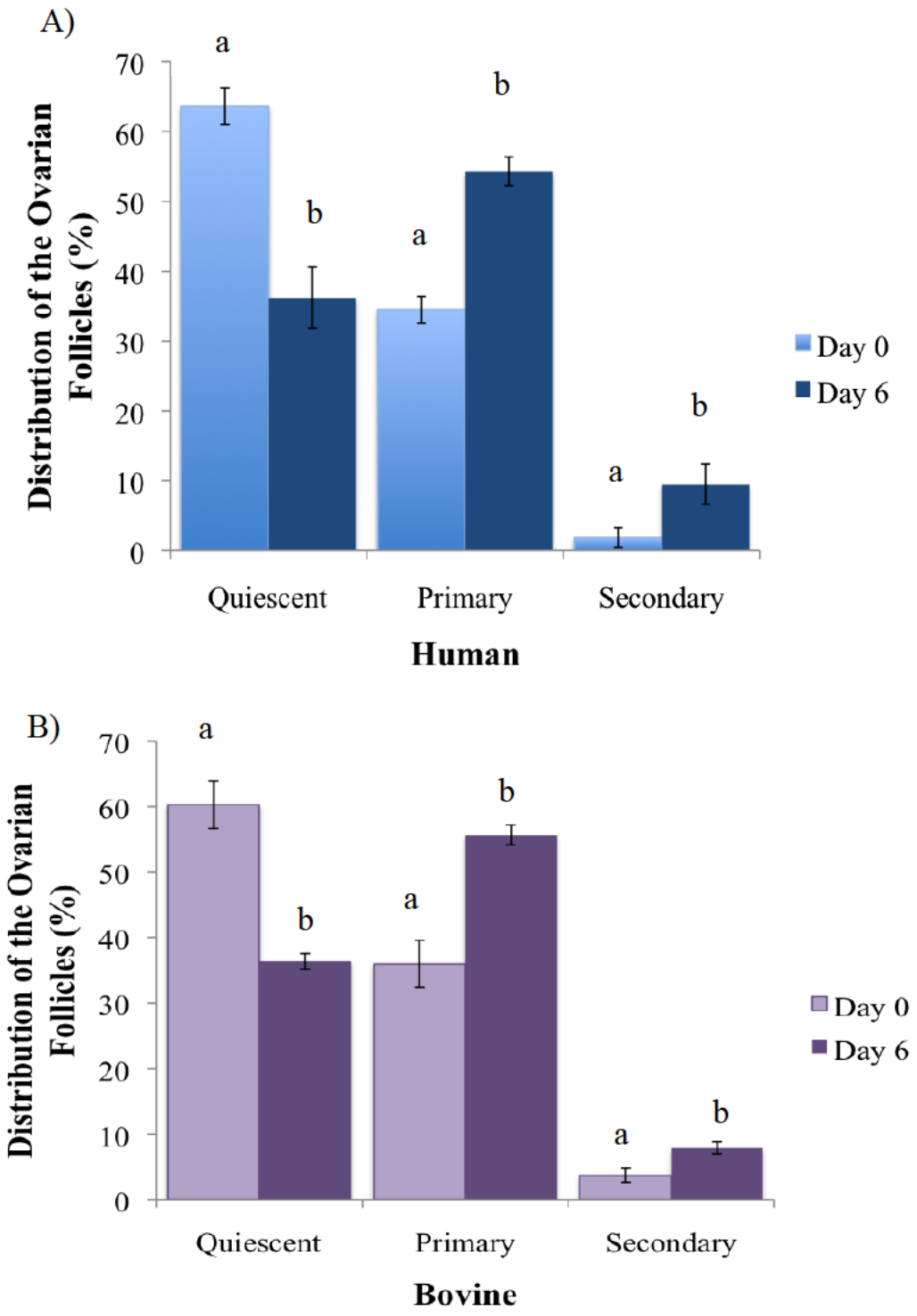


Figure 4.3 Distribution of the Ovarian Follicle Population in the Human and Bovine. The two graphs show the follicular distribution from day 0 to day 6 in the A) human and the B) bovine. Means that have different letters are significantly different from one another within quiescent, primary and secondary follicle populations. In both the human and the bovine there is a significant decrease in the quiescent follicle population ($p<0.001$) and an increase in the primary ($p<0.001$) and secondary ($p<0.05$) follicle populations from day 0 to day 6. Mean±sem, n=10, human; n=12 bovine.

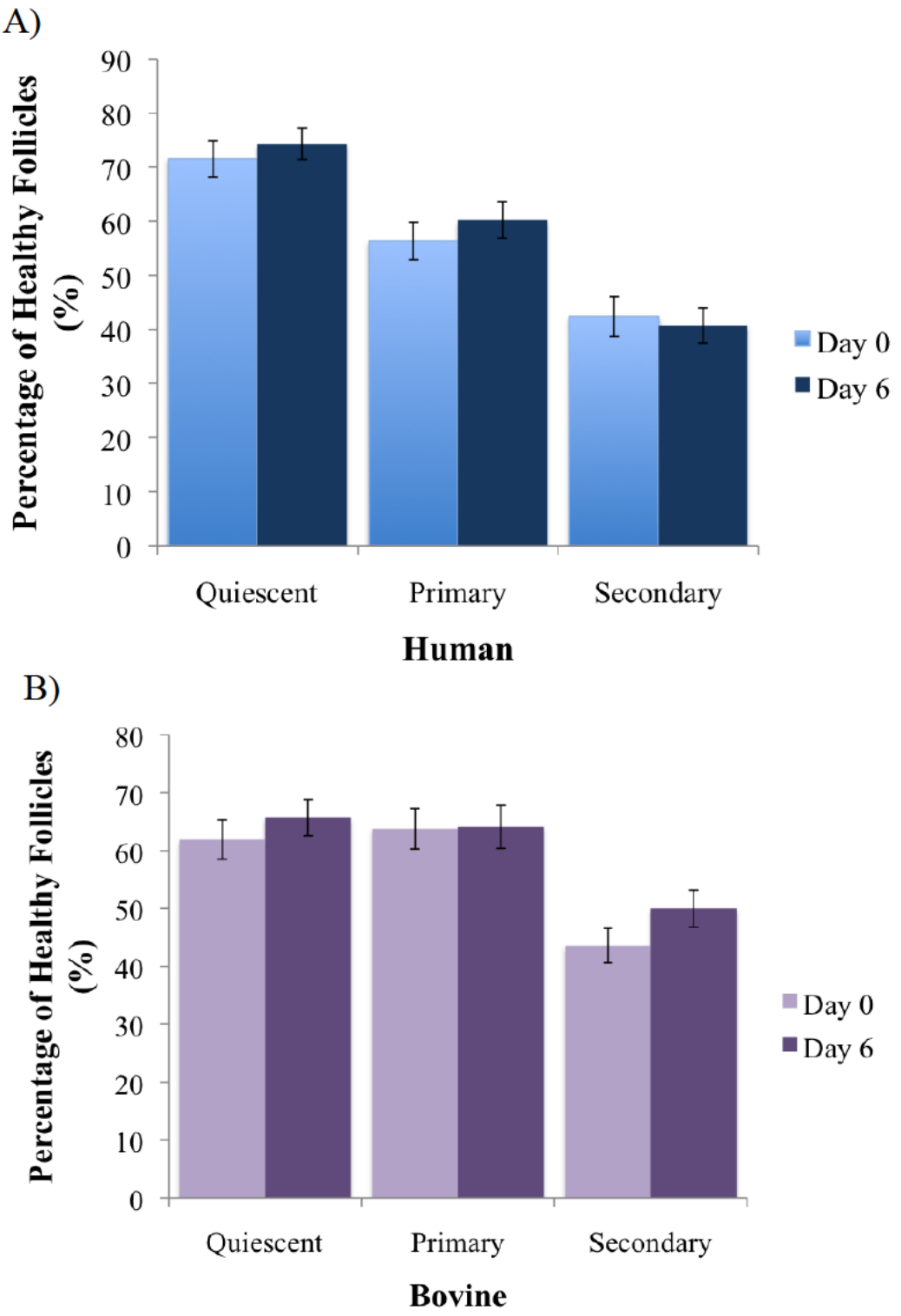


Figure 4.4 Health of the Ovarian Follicle Populations in the Human and Bovine Shows the proprotion of healthy of follicle in the queiscent, primary and secondary follicle populations between the uncultured day 0 samples and cultured day 6 samples in the A) human and B) bovine. In both the human and the bovine tissue there is no significant difference in the health of the ovarian follicles at any development stage between day 0 and day 6 ($p>0.05$). Mean±sem, n=10, human; n=12 bovine.

4.3.2 Comparing Human and Bovine Quiescent Follicle Activation and Growth within the *In Vitro* Culture System

In the human and the bovine the distribution of the ovarian follicles between at day 0 was seen to be similar with 63.6±4.2% and 59.6±5.5% quiescent ($p>0.05$), 34.5±2.9% and 54.3±4.4% primary ($p>0.05$) and 1.9±1.0% and 2.0±1.3% secondary ($p>0.05$) in the human and the bovine respectively (see figure 4.3). The distribution of the ovarian follicles was also seen to be similar at day 6 in the human and bovine, with 36.2±5.2% and 36.3±4.9% quiescent ($p>0.05$), 54.3±4.4% and 55.8±2.9% primary ($p>0.05$) and 9.5±3.4% and 7.9±1.9% secondary ($p>0.05$) in the human and bovine respectively.

The rate of activation was measured as a percentage change in the proportion of the ovarian follicles that were part of the growing follicle population from day 0 to after 6 days in culture. Between the human and the bovine there was a similar level of activation after 6 days in culture at 26.7±5.7% in the human and 23.5±4.3% in the bovine ($p>0.05$) (see figure 4.5 A).

The activation of ovarian follicles led to an increase in the growing follicle population which consisted of both primary and secondary ovarian follicles. In both the human and bovine the populations of primary and secondary follicles increased by similar proportions (see figure 4.5 B). In the two species the primary follicle population increased after culture *in vitro* by 19.9±4.4% and 17.1±3.8% ($p>0.05$) in the human and bovine respectively. The secondary follicle population in both the bovine and human increased by similar amounts at 7.9±4.9% and 5.6±3.2% ($p>0.05$) respectively after being cultured *in vitro*.

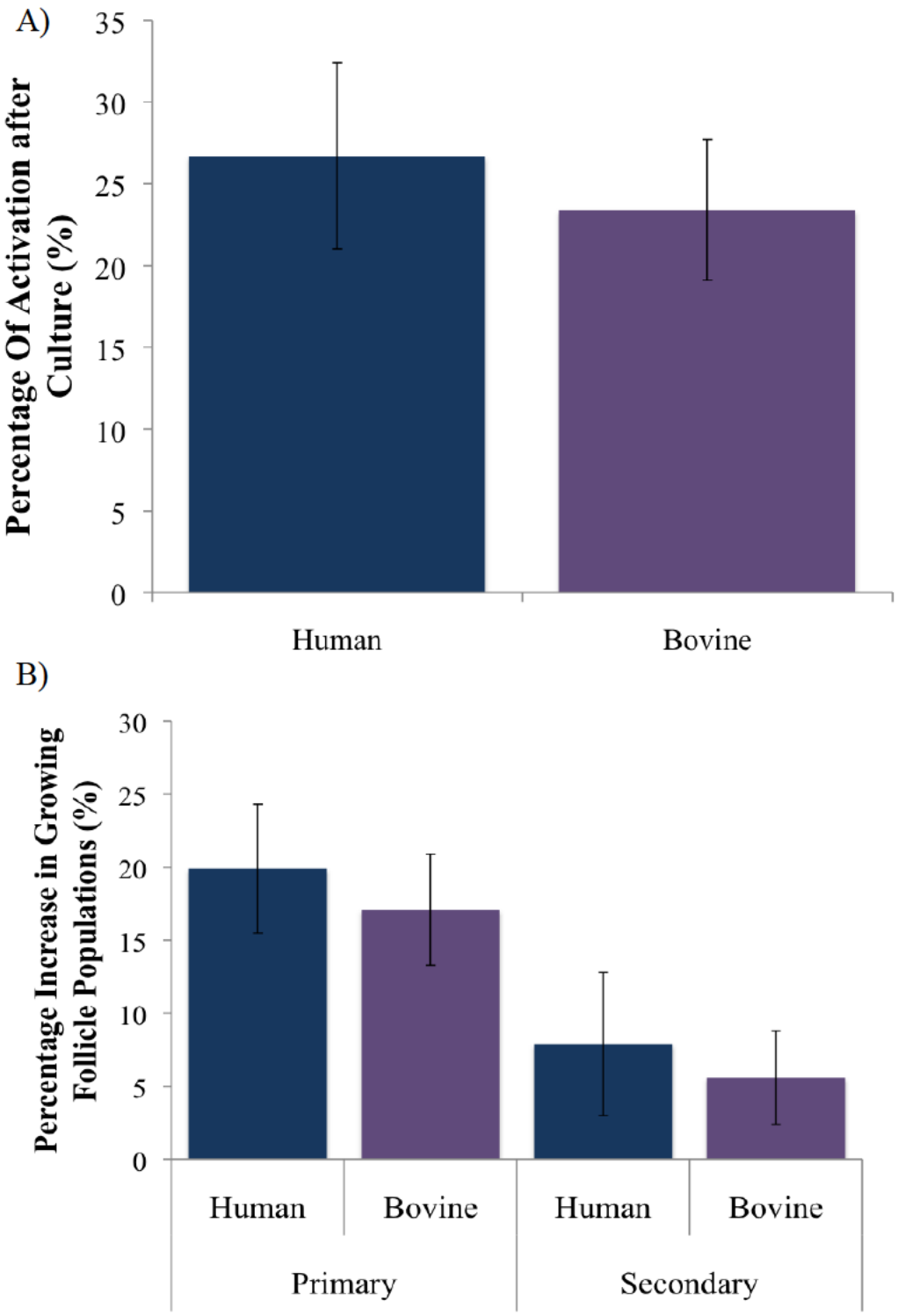


Figure 4.5 Follicular Activation and Increase in the Growing Follicle Populations. A) Displays the levels of activation in both the human and the bovine, no significant difference in the level of activaion was observed between the two species ($p>0.05$). B) Displays the increase in the primary and secondary follicle populations again no significant difference in the increase of these populations was observed in the human or the bovine ($p>0.05$). Mean±sem, n=10, human; n=12 bovine.

4.3.3 Follicle Growth

The diameters of the quiescent and primary follicles were compared between day 0 and day 6 samples from both bovine and human. In the bovine the quiescent follicles had a mean diameter of 23.9 ±0.3μm at day 0 (n=205) and 23.6 ±0.2μm at day 6 (n=125) ($p>0.05$) and the primary follicles had a mean diameter of 29.1 ±0.2μm at day 0 (n=138) and 29.4 ±0.2μm at day 6 (n=193) ($p>0.05$). In the human the quiescent follicles had a mean diameter of 35.7 ±0.2μm at day 0 (n=108) and 35.9 ±0.3μm at day 6 (n=75) and the primary follicles in the human had a mean diameter of 45.2 ±0.3μm at day 0 (n=63) and 45.6 ±0.2μm at day 6 (n=112) ($p>0.05$). To examine the growth of the quiescent follicles to become primary follicles the mean increase in follicle diameter was compared between the human and the bovine. In the human there was a statistically larger increase in the diameter of the ovarian follicle from the quiescent to primary stage of development at 9.8 ±0.8μm diameter increase compared to the bovine were there was a diameter increase of 5.5 ±0.6μm ($p<0.05$) (see figure 4.6 A).

The diameter of the quiescent and primary follicle oocytes were compared between day 0 and day 6 from both bovine and human. In the bovine the quiescent follicle oocytes had a mean diameter of 20.2 ±0.2μm at day 0 (n=205) and 20.3 ±0.3μm at day 6 (n=125) ($p>0.05$) and the primary follicle oocytes in the bovine had a mean diameter of 24.3 ±0.3μm at day 0 (n=138) and 24.5 ±0.2μm at day 6 (n=193) ($p>0.05$). In the human the quiescent follicle oocytes had a mean diameter of 33.6 ±0.3μm at day 0 (n=108) and 33.9 ±0.2μm at day 6 (n=75) and the primary follicle oocytes in the human had a mean diameter of 37.9 ±0.3μm at day 0 (n=63) and 38.1 ±0.2μm at day 6 (n=112) ($p>0.05$). To examine the growth of the oocytes in the quiescent follicles to become primary follicles the mean increase in oocyte diameter was compared between the human and the bovine. There was no statistical difference in the increase in the diameter of the oocyte between human and bovine from the quiescent to the primary stage of development at 4.6 ±0.3μm and 4.2 ±0.3μm respectively (see figure 4.6 B).

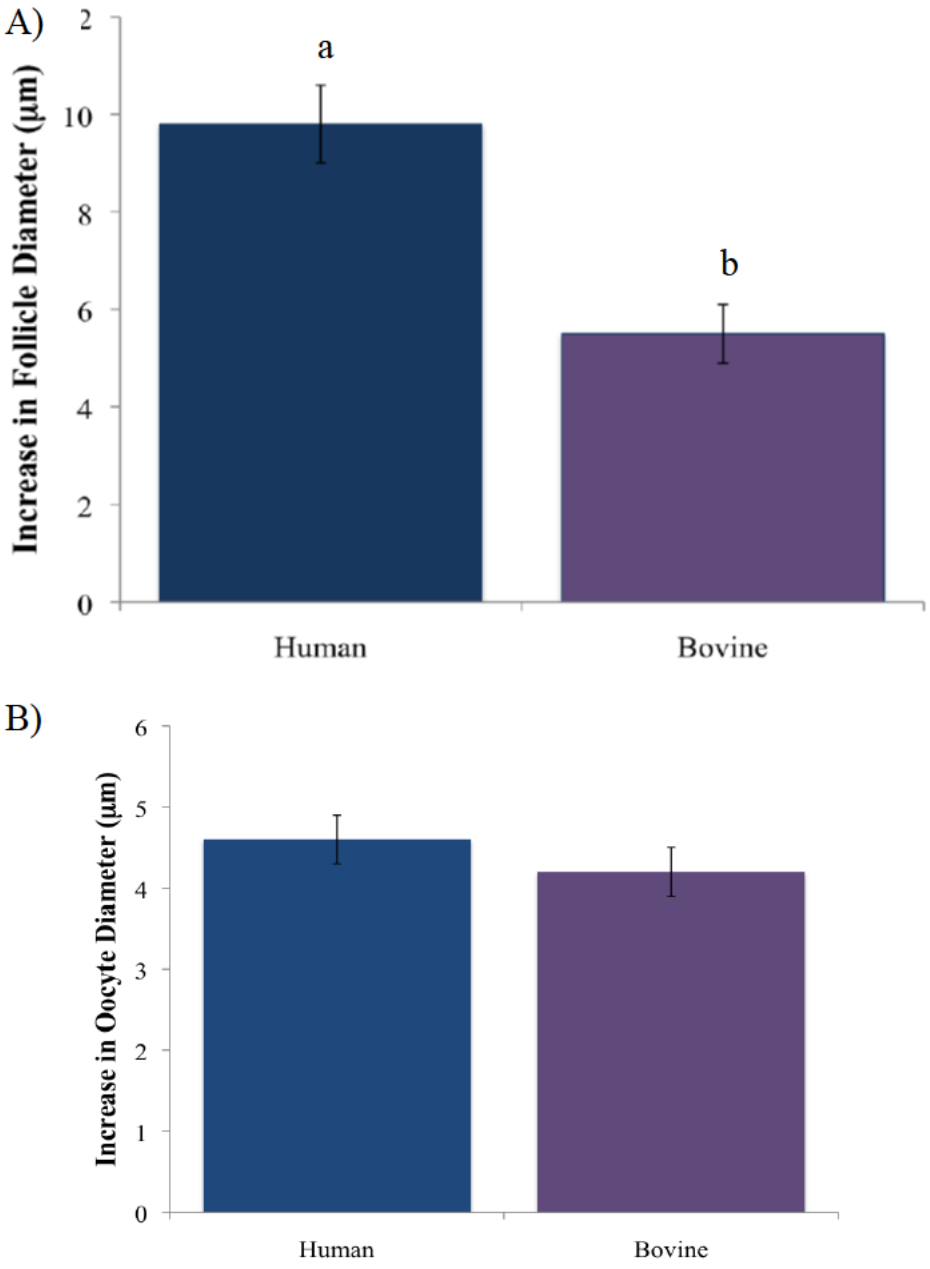


Figure 4.6 Diameter Increase in the Ovarian Follicle and Oocyte from Quiescent to Primary Stage of Development in the Bovine and Human. A) Displays the increase in diameter of the ovarian follicles between the quiescent and primary stage of development in both the human and the bovine. Means that have different letters are significantly different from one another ($p<0.05$). The human was shown to have a significantly larger increase in diameter in comparison to the bovine ($p<0.05$). B) Displays the increase in the size of the oocyte between the human and the bovine, here no significant difference was observed between the two species ($p>0.05$). Mean±sem, n=10, human; n=12 bovine.

4.3.4 Concentration of Ovarian Follicles

There was no significant difference in the concentration of ovarian follicles between the day 0 and day 6 samples in either the human or bovine ($p>0.05$) (see figure 4.7). However, there was a significantly higher concentration of ovarian follicles in the bovine with 148.4±19.5 ovarian follicles/ mm^3 compared to the human with only 13.4±4.12 ovarian follicles /mm^3 ($p<0.01$).

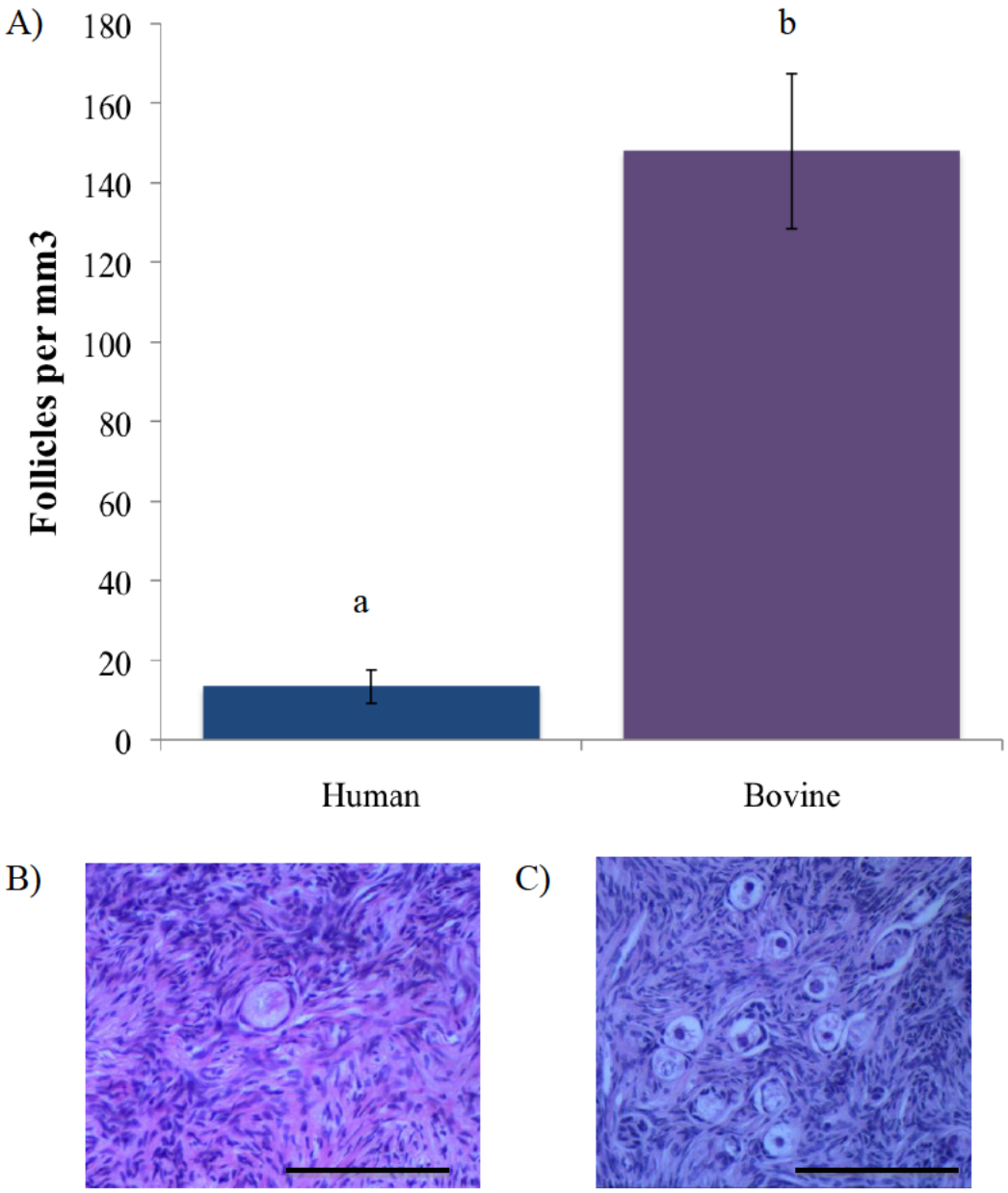


Figure 4.7 Concentration of Ovarian Follicles. A) Displays the difference in the concentration of ovarian follicles in the ovarian tissue of the human (n=10) and bovine (n=12). Means that have different letters are significantly different from one another ($p<0.01$). The bovine has a significantly higher concentration of ovarian follicle in comparison to the human ($p<0.01$). This is demonstrated in the B) human in comparison to the C) bovine where there are usually more ovarian follicles. Mean±sem, n=10, human; n=12 bovine. Scale bar in B and C is equal to 90µm.

4.4 Discussion

The aim of this study was to establish if the bovine is a good model for human folliculogenesis in an *in vitro* culture system. This was achieved by culturing both human and bovine *in vitro* and comparing the level of activation, the changes in the proportion of the primary and secondary follicle populations and the increase in follicle and oocyte diameters of the quiescent and primary follicles between the bovine and human. The data showed that the culture system promoted the activation of the quiescent follicles to become growing follicles for both the bovine and the human and that the follicles maintained their health within the culture system, agreeing with the data in the Telfer et al 2008 and McLaughlin and Telfer 2010 studies.

In vivo the development of a quiescent follicles to become a secondary follicle is known to take up to 120 days in the human (Gougeon, 1986) and 160 in the bovine (Russe, 1983). Early follicular growth and development is thought to be extremely protracted (Telfer, 1998; Telfer and McLaughlin, 2011) however, it is unclear if the time taken *in vivo* is the definitive rate of the development for ovarian follicles or whether growth *in vivo* is modulated by local constraints. In this chapter a significant proportion of the quiescent follicles became part of the growing follicle population in both bovine and human after 6 days of culture. A significant number of quiescent follicles joining the growing follicle population have been previously observed in short term cultures in both the human (Telfer et al., 2008; Hovatta et al., 1997; Wright et al., 1999) and the bovine (McLaughlin and Telfer, 2010; Wandji et al., 1996b), suggesting that activation occurs as a result of a release of intraovarian factors that normally inhibit the activation (Adikari and Liu, 2009; Wandji et al., 1998). One cause could be the removal of the medulla, as the medullary region of the ovary is thought to secrete inhibitory factors to slow down ovarian follicle growth (Fortune et al., 2000), highlighted by activation in the whole ovarian cultures being more limited in comparison to the ovarian strips where the medulla has been removed (Eppig, 1996; Wandji et al., 1996b). Another factor could be disruption of Hippo signalling. Hippo signalling pathway is important in maintaining optimal organ size (Pan, 2007; Halder and Johnson, 2011, Hergovich, 2012). Dissecting the

tissue into smaller pieces in the murine ovaries promoted actin polymerization that disrupts Hippo signalling, which resulted in an increased expression of down stream growth factors and the promotion of follicular growth (Kawamura et al., 2013). Therefore, dissecting the human and bovine ovarian sample into small fragments could be disrupting the Hippo signalling resulting in follicle growth and thereby an increase in the proportion of ovarian follicles becoming part of the growing follicle population.

The activation of the primordial follicles causes a change in the ovarian follicle's morphology, characterised by the enlargement of its oocyte and the flattened granulosa cells becoming cuboidal as they transform into primary follicles (McGee and Hsueh, 2000; Picton, 2001). The bovine and the human characteristically have approximately the same range of ovarian follicle diameters for each of the different developmental stages (see figure 4.1) (Braw-Tal and Yossefi, 1997; Gougeon, 1996), suggesting that the ovarian follicles must grow roughly by the same amount as they transition between various stages of folliculogenesis. However, in this study it was observed that the human ovarian follicles had a larger percentage increase in diameter from quiescent stage to the primary stage of development compared to the bovine, indicating that human ovarian follicles undergo more growth to reach the primary stage from the quiescent stage of development. However, this difference could be due to the varying levels of primordial and transitional follicles within the quiescent follicle populations of the two species. The majority of the quiescent follicles population in the bovine model are transitional follicles (vanWezel and Rodgers, 1996b), whereas in the human the transitional population only makes up only half of the quiescent follicle population (Gougeon and Chainy, 1987). Therefore, the difference in the percentage increase in the diameter of the ovarian follicles could be attributed to the varying number of cuboidal granulosa cells, which are larger in size in comparison to the flatter granulosa cells. The difference in the proportion of the primordial and transitory follicles within the two species could therefore, be influencing the observed increase of the ovarian follicle diameter during the transition between the quiescent and primary stages of development.

Between the human and the bovine the oocyte is also seen to be similar in diameter at each stage of ovarian follicle development (Braw-Tal and Yossefi, 1997; Gougeon, 1996). Therefore, to remove the impact of the different proportions of primoridal or tranisitory follicles in the quiescent follicle population in the bovine and human, the oocytes of both the quiescent follicles and primary follicles were examined. The oocytes were examined to see if there was a difference in the oocyte diameter during the transition between the quiescent and primary stage of development in the bovine and human. It was observed that the oocyte diameter in both the human and the bovine increased by a comparable amount from the quiescent to the primary stage of development, indicating that a similar amount of growth must occur in the oocyte during this transition from the quiescent to the primary stage of development in both species.

The distribution of the ovarian follicles is known to vary widely in the ovary with around 90% of the ovarian follicles found within the cortex, and even within this region the ovarian follicles are not evenly distributed (Jimenez, 2010; vanWezel and Rodgers, 1996), therefore, it is important to note that the concentrations of the ovarian follicles seen in figure 4.7 are based on the follicle concentration in the cortex rather than the ovary as a whole. However, as it is the cortex that is utilised in both fertility preservation (von Wolff et al., 2009) and *in vitro* research examining primordial follicles (Braw-Tal and Yossefi, 1997; McLaughlin and Telfer, 2010; Telfer et al., 2008; Wandji et al., 1996a; Wandji et al., 1997) in species where culturing the whole ovary is not possible due to its large size (Telfer et al., 2000; Thomas et al., 2003; Wandji et al., 1996b), comparing the cortex of the two species is beneficial in determining if the bovine is a good model for the human follicle activation *in vitro*. The concentration of ovarian follicles within the cortex is much higher in the ovarian samples from the bovine than those from the human. This difference in concentration can be attributed to the difference in the age at which the samples were collected from the two species. Age is an important factor in determining the number of ovarian follicles left within the ovary, as the number declines with increasing age as the primordial follicles are activated (Erickson, 1966, Gosden and Telfer, 1987a; Gosden and Telfer, 1987b; Wallace and Kelsey, 2010). It has previously been observed that ovarian tissue collected from women over the age

of 30 have a much lower density of primordial follicles in comparison to younger ages which makes them less useful for testing a range of concentrations within drug treatments or optimising conditions (Cortvrindt and Smitz, 2001b; Lass et al., 1997). Caesarean section patients are the most abundant source of ovarian samples for analysis in our laboratory, and are generally above the age of 25. Using the bovine with its higher concentration of primordial follicles would be beneficial, as a model, to optimise the culture conditions or to select drug treatment concentrations before testing it in the human to ensure that the human tissue is used in the best way.

The evidence from this study indicates that the bovine is a good model for human primordial follicle activation and subsequent growth in an *in vitro* culture system. This is due to a number of similarities seen between the two species including comparative levels of activation and subsequent increases in the primary and secondary follicle populations. There was also seen to be a similar amount of growth required for the both to progress from quiescent to the primary stage of development observed in the diameter of the oocyte. The higher concentration of ovarian follicle in the bovine samples makes the bovine a good model, as this will allow the culture conditions and selected drug treatment concentrations to be optimisation before testing in human ovarian sample.

Chapter Five:
The Role of the PI3K Pathway in Primordial Follicle Activation.

5.1 Introduction

It is widely accepted that the female's entire store of oocytes is formed in fetal or early life dependent on the species, with no additional oocytes created in later life (McGee and Hsueh, 2000; Zuckerman, 1951). The store of mammalian oocytes is held in an immature and dormant state as primordial follicles (Escobar et al., 2011; McGee and Hsueh, 2000). In order for mature oocytes to be produced the primordial follicles must be activated to undergo folliculogenesis. Folliculogenesis is the process of ovarian follicle development in which a recruited primordial follicle grows and develops until it reaches a point where the oocyte can be matured (Binelli and Murphy, 2010; McGee and Hsueh, 2000). Activation of primordial follicles is irreversible so once activated each follicle will continue through the various stages of folliculogenesis or undergo atresia (Elvin and Matzuk, 1998). The female's reproductive lifespan is therefore, dependent on the number of dormant primordial follicles remaining within the ovary and so the loss of the primordial follicles from the dormant pool through activation must be tightly controlled or the individual will undergo POI.

The mechanisms involved in controlling primordial follicle activation are still unclear, however a number of different studies have highlighted a range of factors that appear to play a role (Adhikari et al., 2013; Kim, 2012; Monget et al., 2012; Pangas, 2012; Reddy et al., 2010). Many of these factors are part of the PI3K pathway, for example Pten (Reddy et al., 2008), Foxo3 (John et al. 2008, Castrillon et al 2003, Liu et al., 2007), Pdk1 and rps6 (Reddy et al., 2009) (see figure 5.1). This pathway is known to play an important role in cellular survival, growth and proliferation through a variety of downstream mechanisms (Cantley, 2002; Stokoe, 2005) and is considered to be one of the key pathways involved in both primordial follicle activation and maintaining the dormancy of the primordial follicle pool (Castrillon et al., 2003; John et al., 2008; Li et al., 2010; Liu et al., 2007; McLaughlin et al., 2014; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008).

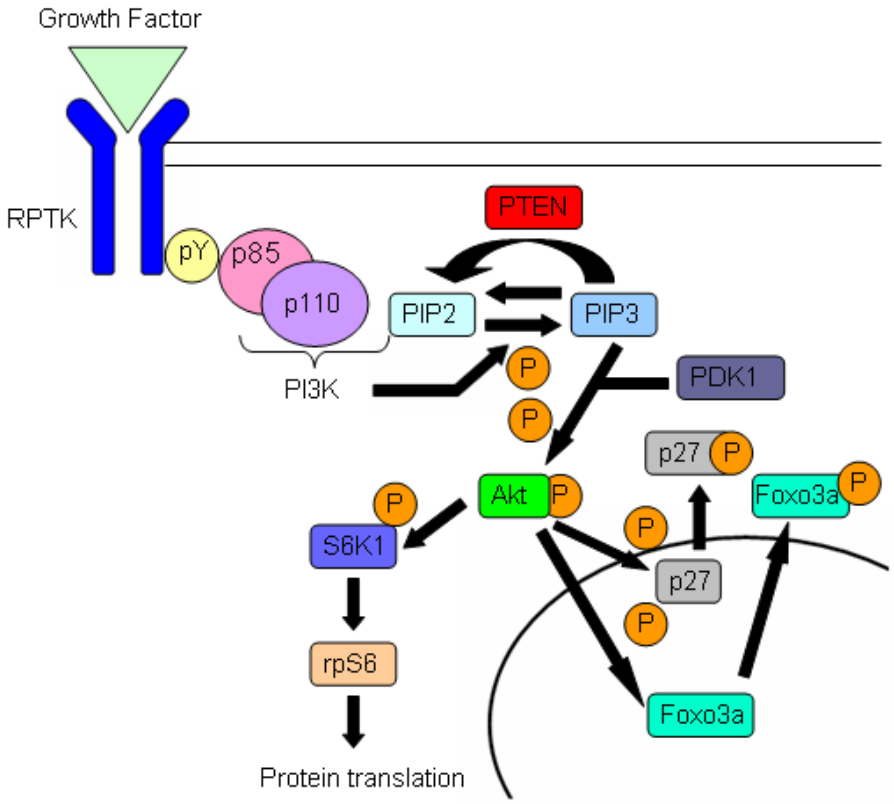


Figure 5.1: The PI3K Pathway. This diagram displays the details of the different factors that are involved in PI3K pathway and each of the key phosphorylation steps that occur during the stimulation of the PI3K pathway. These factors include Pten, Foxo3, Pdk1 and rps6 all of which have been shown in previous studies to play a role in controlling primordial follicle activation and maintaining the dormancy of the primordial follicle pool.

The Reddy et al., 2008 and Reddy et al., 2009 studies are good examples for demonstrating the role of the PI3K pathway in primordial follicle activation and dormancy. The Reddy et al., 2008 study deleted the Pten gene from the oocytes of mice by crossing $Pten^{loxP/loxP}$ mice (Groszer et al., 2001) with transgenic mice expressing Gdf-9 promoter-mediated Cre recombinase (Lan et al., 2004). Pten negatively regulates the PI3K pathway by converting PIP3 back into PIP2 (Cantley, 2002), without it there is an up-regulation of the PI3K pathway. In the oocyte-specific Pten knockout mouse model there was an increase in primordial follicle activation so by PD8 only 49.6% of the ovarian follicles were primordial in comparison to 83.6% of the ovarian follicles being primordial in the wild type mice. Consequently, by PD35 there were virtually no primordial follicles left in the ovary

in the oocyte-specific Pten knockout mice (Reddy et al., 2008) (see figure 5.2) demonstrating the consequence of widespread primordial follicle activation. The Reddy et al., 2009 study deleted Pdk1 from the oocytes of mice by crossing $Pdk1^{loxP/loxP}$ mice (Hashimoto et al., 2006, Inoue et al., 2006) with transgenic mice expressing Gdf-9 promoter-mediated Cre recombinase (Lan et al., 2004). Pdk1 is required to co-bind with PIP3 to activate Akt thereby activating the downstream components of the PI3K pathway (Engelman et al., 2006; Mora et al., 2004); without it there is a down regulation of the PI3K pathway. By PD35 only 33.7% of the total number of ovarian follicles observed in the wild type ovary were found in the oocytes-specific Pdk1 deficient mice and only 61.6% of the proportion of growing follicles (Reddy et al., 2009). This shows a decrease in primordial follicle activation and the loss of the primordial follicles directly from the primordial follicle pool highlighting their inability to maintain their dormant state.

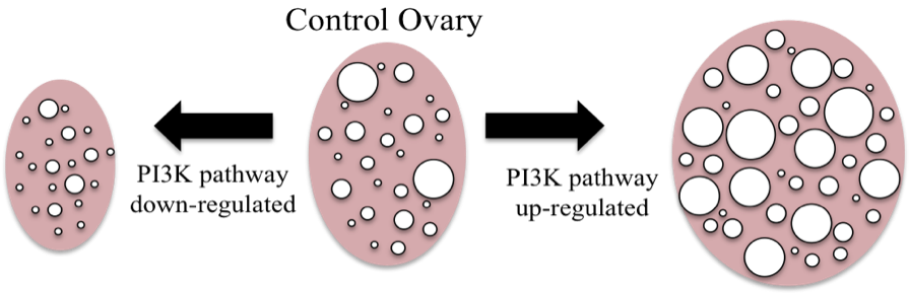


Figure 5.2: Role of the PI3K pathway in Primordial Follicle Activation and Dormancy. This figure displays the impact of changing the level of PI3K within the ovary representing the data found within the previous mouse studies. When the PI3K pathway is down regulated the ovary becomes smaller in size due to it predominately having only quiescent follicles. Contrastingly, when the PI3K pathway is up regulated the ovary becomes larger due to the increased number of activated follicles, which have begun to grow and are therefore, are much larger in size.

The role of the PI3K pathway in primordial follicle activation has mainly been explored by using knockout mouse models (Castrillon et al., 2003; John et al., 2008; Liu et al., 2007; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008) therefore, it is important to establish whether it has the same role in other species. Recently, two pharmacological compounds 740 Y-P and bpV (HOpic) have been

shown to be able up-regulate the PI3K pathway. This has allowed the PI3K pathway to be explored in species where it is not possible to use a knockout model (Li et al., 2010; McLaughlin et al., 2014). 740 Y-P binds to the SH2 domain of the p85 regulatory subunit of PI3K inhibiting its inhibition of p110 thereby, stimulating the activity of p110 causing an up-regulation of the PI3K pathway (see figure 5.3) (Derossi et al., 1998). bpV (HOpic) binds to PTEN inhibiting its ability to convert PIP3 back into PIP2 again resulting in an up-regulation of the PI3K pathway (Bevan et al., 1995; Li et al., 2010; Posner et al., 1994; Schmidt et al., 2004). 740 Y-P and bpV (HOpic) caused a similar increase in primordial activation in the mouse model to the knockout mouse models where PI3K pathway had been up regulated (Li et al., 2010).

The role of the PI3K pathway has been explored in the human using bpV (HOpic) (Li et al., 2010; McLaughlin et al., 2014); these studies demonstrate an increase in primordial follicle activation but resulted in a decrease in follicle survival. However, there is still much we do not know about the role of the PI3K pathway in large mono-ovulatory species like the human. This study will explore the hypothesis that in the bovine model, both bpV (HOpic) and 740 Y-P will cause an up-regulation of the PI3K pathway thus causing increased primordial follicle activation, as observed in both the mouse and human models (Li et al., 2010; McLaughlin et al., 2014). This chapter aims to examine the role of the PI3K pathway on follicle activation, health and development, using both bpV (HOpic) and 740 Y-P in a range of concentrations and to explore the impact of different levels of stimulation and the way the PI3K pathway is up-regulated on the ovarian follicle population.

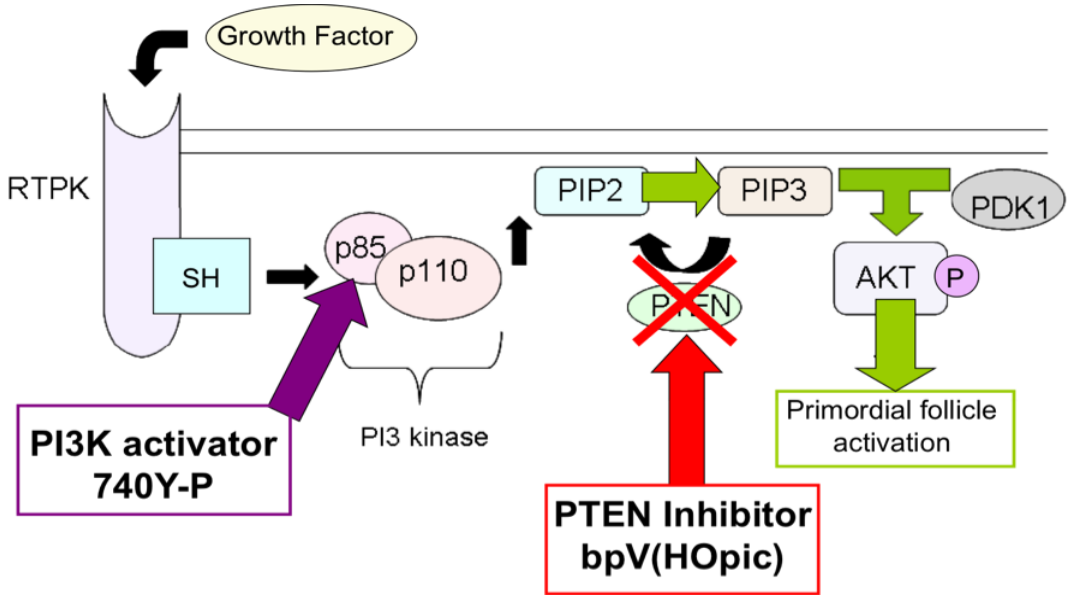


Figure 5.3: Interactions of 740 Y-P and bpV (HOpic) with the PI3K pathway. 740Y-P binds to the p85 subunit of PI3 kinase to inhibit its inhibition of p110 thereby, increasing the catalytic activity of p110. bpV (HOpic) inhibits PTEN preventing it from converting PIP3 back into PIP2. Both cause the up-regulation of the PI3K pathway resulting in increased levels of phosphorylated Akt and thereby increased primordial follicle activation.

5.2 Materials and Methods

5.2.1 Tissue Collection and Dissection

Bovine ovaries were collected from local abattoir from animals aged between 10-14 months and transported to the laboratory in supplemented M199 medium. Strips of the cortex were removed from the ovaries to a depth of 1-2mm. The strips were examined under a light microscope in bovine Leibovitz to ensure that all the ovarian follicles within them were less than 40μm in diameter. These cortical strips were then dissected to approximately 0.5-1 mm^2 in size (as described in chapter 2).

5.2.2 Exploring the Levels of Akt and p-Akt

5.2.2.1 Tissue Treatment

Dissected cortical strips (see section 5.21) were cultured for 24 hours in bovine McCoy's (see figure 5.4). All medium was then removed and the cortical strips were treated with either fresh bovine McCoy's, bovine McCoy's with 10μM bpV (HOpic) or bovine McCoy's with 100μg/ml 740 Y-P for 15 minutes (see figure 5.4). The samples were then snap frozen for protein extraction and western blotting.

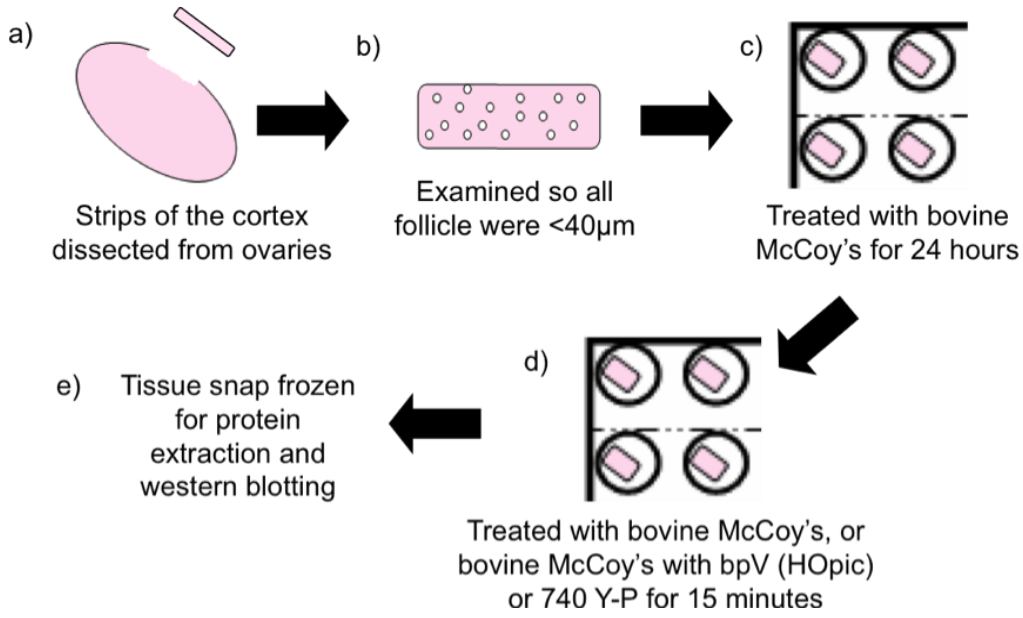


Figure 5.4: Tissue Collection for Western Blotting. a) Strips of the cortex were taken from the bovine ovaries and b) examined to ensure all ovarian follicles were less than 40µm in diameter. c) All cortical strips were incubated in bovine McCoy's for 24 hours. The cortical strips were then d) treated with bovine McCoy's or bovine McCoy's with either bpV(HOpic) or 740 Y-P for 15 minutes. All cortical strips were then immediately e) snap frozen for protein extraction and western blotting.

5.2.2.2 Protein Extraction and Western Blotting

Protein was extracted using a hand held homogeniser in RIPA buffer which consisted of 25mM Tris HCl, 150mM NaCl, 1% Triton x-100, 0.1% SDS and 0.05% sodium deoxycholate. For every 10ml of RIPA buffer 1 mini-complete protease inhibitor cocktail tablet and 1 mini-phospho-STOP tablet was added (Roche Products Limited, Welwyn Garden City, UK). Once homogenised the samples were spun at 14000g for 10 minutes in a centrifuge. The concentration of protein within each sample was established by comparing its absorbency to standard BSA (Thermo Fisher Scientific, Hemel Hempstead, UK) concentrations of 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0mg/ml. Each assay contained 5µl of a 1/10 dilution of the sample or BSA concentrations along with 25µl of Reagent A*; which consists of 20µl of Reagent S to every 1ml of Reagent A, and 200µl of Reagent B, all reagents are from a western blotting kit from Corning Costar Europe, in a flat bottomed clear 96-well plate (Corning Costar Europe). All samples had three repeats. The samples

were placed on a plate shaker at room temperature for 15 minutes, and then the absorbance measured at 590nm on a plate reader. Concentrations of the protein samples were calculated by comparing their absorbencies against the absorbencies of the known BSA standard concentrations.

For each treated piece of tissue two protein samples were used. The protein samples were prepared so each sample contained 20µg of protein plus RIPA buffer to give a total volume of 20µl. Each sample also contained 5µl of SDS Sample Buffer consisting of 6.25 Tris with 5% glycerol, 2% SDS and 10% water and 10% β-mercaptoethanol. Samples were heated to 99°C for 6 minutes then cooled on ice. Each sample was then loaded into individual pre-packed 4-20% gels (Thermo Fisher Scientific) alongside 5µl Page Ruler Plus ladder (Thermo Fisher Scientific, Hemel Hempstead, UK). The loaded gels were run in 1×Tris-HEPES SDS Buffer (Thermo Fisher Scientific) at 125V for 1 hour. Immobilon FL blotting paper (one per gel) (Merck Millipore, Nottingham, UK) was rinsed in methanol for 30 seconds then water for 1 minute and finally 1× Semi-Dry Fast Transfer Buffer (Thermo Fisher Scientific) for 15 minutes. Two pieces of blotting paper were cut per gel and soaked in the 1× Semi-Dry Fast Transfer Buffer before being use. The gel and Immobilon FL blotting paper were sandwiched between two pieces of blotting paper in a Pierce Fast Semi-Dry Blotter unit (Thermo Fisher Scientific) for 9 minutes at 25V.

The Immobilon FL blotting paper now with the protein on it (from here on referred to as the blot) was rinsed in water (2 × 5 minutes) then washed with phosphate buffered saline with 0.1% Tween (PBST) (4 × 5 minutes). The blot was then placed in a block solution consisting of a 1:1 solution of blocking buffer (Rockland Immunochemicals Inc. PA, USA) and 0.1% PBST at room temperature. After this the blot was incubated with a rabbit polyclonal antibody raised against either Akt (9272) or pAkt (9271) (Cell Signalling, Hitchin, Hertfordshire, UK) both diluted to 1:1000 and a mouse monoclonal antibody raised against β-actin (A5441) (Sigma-Aldrich) diluted to 1:3000. The primary antibodies were left on the membrane in a cold room at 4°C overnight. The blot was then washed in 0.1% PBST (4 × 5 minutes), then incubated with a polyclonal donkey antibody raised against rabbit IgG (heavy and light chain) conjugated with Alexa Fluor680 (Invitrogen, Paisley, UK) and a donkey polyclonal antibody raised against mouse IgG (heavy and

light chain) conjugated with IRDye8000 (Rockland Immunochemical) both diluted to 1:10000 for an hour at room temperature. Before scanning the blot was washed in 0.1% PBST (2 × 5 minutes) then in 0.1% PBS (2 × 5 minutes). The membrane was then scanned using a LI-COR Odyssey Infrared Imagine device (LI-COR Biosciences UK Ltd, Cowley Road, Cambridge, UK) to detect the fluorescent bands and calculate the concentration of each band for analysis.

5.2.3 Bovine Tissue Culture

Dissected cortical strips (see section 5.21) were treated with bovine McCoy's or bovine McCoy's with 1µM, 10µM or 100µM of bpV (HOpic) or 0.1µg/ml, 1µg/ml, 10µg/ml or 100µg/ml of 740 Y-P in a humidified incubator at 37°C with 5% CO_2 for 24 hours (see figure 5.5). After 24 hours all the medium was removed and replaced with bovine McCoy's medium only for a further 6 days, with a medium change every 48 hours. The bovine ovaries were then fixed in formalin, processed and placed onto slides for H&E staining (as described in chapter 2).

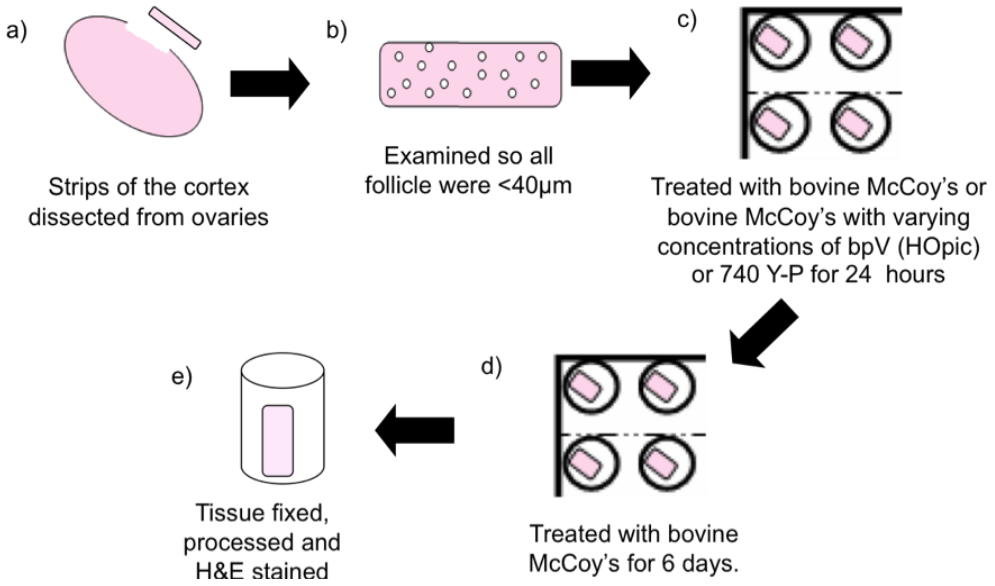


Figure 5.5: Tissue Culture Method. a) Strips of the cortex were dissected from the ovary. b) These cortical strips were further dissected so all ovarian follicles were less than 40µm in diameter. Cortical strips were then treated with c) bovine McCoy's or bovine McCoy's with varying concentrations of bpV (HOpic) and 740 Y-P for 24 hours. d) All the medium was then removed and tissue was cultured for a further 6 days with bovine McCoy's medium only.

5.2.4 Analysis

The western blot data was analysed by initially normalising the Akt and p-Akt bands against the β-actin band. The levels of Akt that had been phosphorylated could thus be established within each sample and compared between the tissue treated with bovine McCoy's to tissue treated with either bovine McCoy's with bpV (HOpic) or bovine McCoy's with 740 Y-P.

Ovarian cortical samples were H&E stained for morphological analysis. The morphology of the ovarian follicles allowed for each of them to be classified as either quiescent, which included the primordial and transitory follicles, or as primary or secondary follicles. Each ovarian follicle was classified as healthy or unhealthy, again based on morphology. Ovarian follicles were also measured using light microscopy with an eyepiece graticule. The ovarian follicle concentration was calculated using ImageJ 1.44g (Rasband W.S, National Institutes of Health, USA, http://imagej.nih.gov/ij/). Full parameters for classification of the ovarian follicles developmental stages, follicular health and calculation of concentration can be seen in chapter 2.

5.2.5 Statistical Analysis

Kolmogorov Smirnoff test was used to test for normal distribution of data, further statistical tests were chosen based on whether the data was normal distributed or not. The bpV (HOpic) and 740 Y-P treatment groups were collected separately with their own control, for both treatments 3 samples were collected from three different experiments to explore the level of Akt and p-Akt (n=3). The levels of phosphorylated Akt were compared between tissue treated with bovine McCoy's with tissue treated with either bovine McCoy's with bpV (HOpic) or bovine McCoy's with 740 Y-P using a two-sampled t-test.

The bovine tissue culture was repeated 4 times for the different treatments with both bpV (HOpic) or 740 Y-P. Each set of experiments for bpV (HOpic) and 740 Y-P where run seperately with a uncultured and control samples taken for each set of experiments. A total of 24 cortical strips were collected from these 4 experiments for each treatment group (n=24). The number of ovarian follicles within

each cortical strips varied between 11-68, with an average of 30 folicles per cortical strip. The uncultured and the control the samples from the separate bpV (HOpic) and 740 Y-P experiments were compared both seperately (n=24) and together (n=48). The distribution of the developmental stages of the ovarian follicles in each cortical strip was compared between the different treatment groups using a chi-square test. The health of the ovarian follicles within each developmental stage in each cortical strip was also compared using a chi-square test.

In order to compare the concentration of ovarian follicles within the different treatment groups the data were first tested for normal distribution using Kolmogorov Smirnoff test. The data was shown to be normally distributed therefore, a two-sampled t-test was used to compare the concentration of ovarian follicles between the uncultured tissue samples and control cultured tissue samples within each cortical strip, with the uncultured to the control the samples from the separate bpV (HOpic) and 740 Y-P experiments being compared both separately (n=24) and together (n=48). The concentration of the ovarian follicles was compared between the control samples and sample exposed to various concentrations of bpV (HOpic) or 740 Y-P using one-way ANOVA, where this test reported a significant difference the groups were compared individually using a post hoc Tukey test.

To compare the diameter of the ovarian follicles and oocytes from the different stages of follicular development both were first tested for normal distribution using a Kolmogorov Smirnoff test. The Kolmogorov Smirnoff test revealed the data to be normally distributed therefore, the diameter of the ovarian follicles within each developmental stage and their oocytes was compared between the uncultured and the control tissue using a two-sampled t-test. The diameter of the ovarian follicles within each developmental stage and their oocytes was compared between the control and the various concentrations of either bpV (HOpic) or 740 Y-P using a one-way ANOVA, where this test reported a significant difference the groups were compared individually using a post hoc Tukey test.

5.3 Results

5.3.1.2 The Level of Akt of Phosphorylation after Treatment with bpV (HOpic) and 740 Y-P Phosphorylation

The levels of total Akt and Akt that had been phosphorylated were compared between the tissue exposed to either bovine McCoy's with bpV (HOpic) (n=3) or 740 Y-P (n=3) to tissue treated with only bovine McCoy's (from here on refered to as the control) for 15 minutes. The level of total Akt was seen to be similar between those treated with control, bpV (HOpic) or 740 Y-P, however, there was a significant increase of phosphorylated Akt of 2.5 ±0.3 fold after exposure to bpV in comparison to the control (HOpic) ($p<0.05$) (see figure 5.6). Similarly, exposure to 740 Y-P caused a significant 2.1 ±0.4 fold increase in the level of phosphorylated Akt in comparison to the control ($p<0.05$) (see figure 5.7).

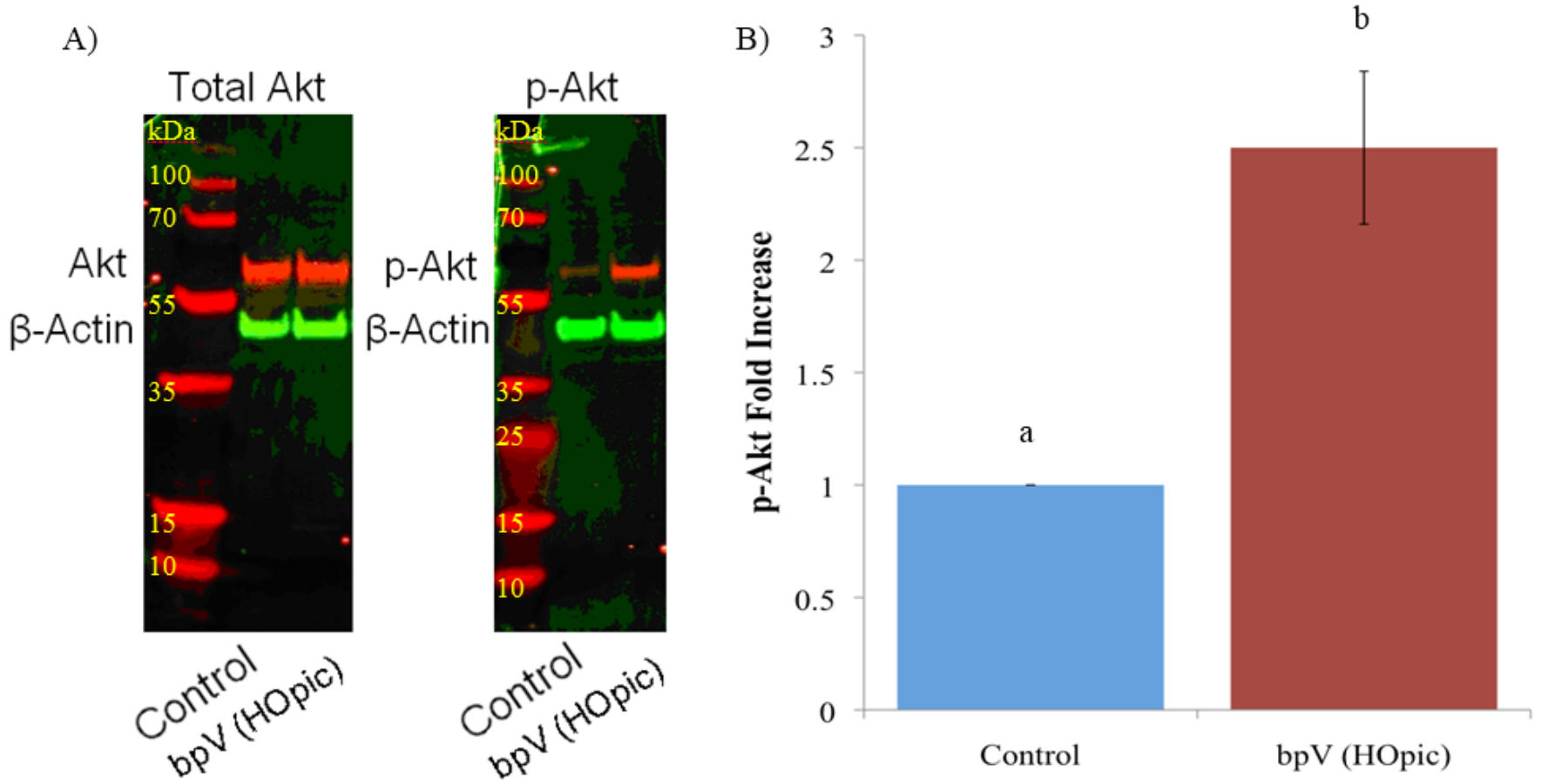


Figure 5.6: Change in the Level of p-Akt after Treatment with bpV (HOpic). Image A) is a representative example of western blotting displaying the levels of Akt and p-Akt in the control and bpV (HOpic) treatment groups. The level of p-Akt is higher after treatment with bpV (HOpic) with a significant B) 2.5 fold increase in comparison to the control. Means that have different letters are significantly different from one another ($p<0.05$).Mean±sem (n=3).

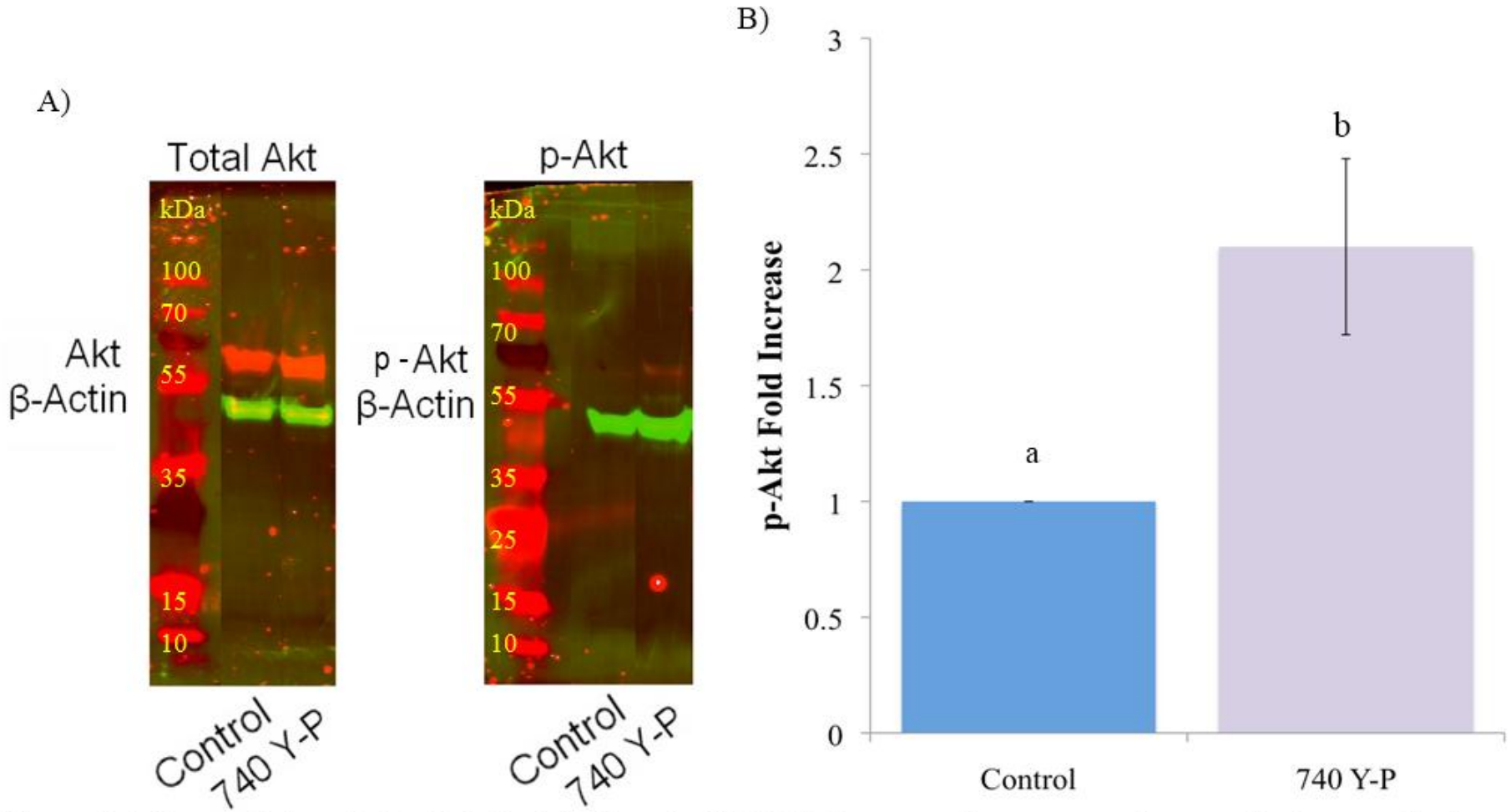


Figure 5.7: Change in Level of p-Akt after Treatment with 740 Y-P. Image A) is a representative example of western blotting displaying the levels of Akt and p-Akt in the control and 740 Y-P treatment groups. The level of p-Akt is seen to be higher after exposure to 740 Y-P to a significant B) 2.1 fold increase. Means that have different letters are significantly different from one another ($p<0.05$). Mean±sem (n=3) ($p<0.05$).

5.3.2 Follicular Distribution after Treatment with bpV (HOpic) and 740 Y-P

The follicular distribution within the cortical strips was examined after treatment with both bpV (HOpic) and 740 Y-P and compared to the control group. Table 5.1 displays a summary of the data collected from the different treatment groups**.** Firstly the distribution of the developmental stages of the ovarian follicles were anaylsed between uncultured tissue and tissue cultured in control medium. The quiescent follicle population decreased significantly from 58.3 ±0.9% in the uncultured samples to only 36.9 ±0.8% in the cultured control samples ($p<0.001$). The primary follicle population significantly increased from 39.8 ±1.0% in the uncultured tissue to 56.9±0.8% in control cultured tissue ($p<0.001$). The secondary follicle population significantly increased from 1.9±0.4% in the uncultured tissue to 6.2±0.4% in control cultured tissue ($p<0.001$) (see figure 5.8).

There was a change in the distribution of the developmental stages of the ovarian follicle populations when comparing the ovarian tissue samples cultured in control medium to those treated in medium containing various concentrations of bpV (HOpic) (see figure 5.9). In control 36.1±1.2% of the ovarian follicles were quiescent which is significantly higher than those treated with bpV (HOpic) at 26.5±1.0%, 25.5±1.3% and 26.0±1.0% in 1µM, 10µM and 100µM bpV (HOpic) respectively ($p<0.001$). No significant difference was seen in the population of quiescent follicle between the different concentrations of bpV (HOpic) ($p>0.05$). In the control tissue 57.5±1.2% of the ovarian follicles were primary follicles; there was a significant increase in the proportion of primary follicles when treated with bpV (HOpic) to 69.6±1.2%, 66.5±1.7% and 66.7±1.0% in 1µM, 10µM and 100µM bpV (HOpic) respectively ($p<0.001$). Again no significant difference was observed in the population of primary follicles between any of the bpV (HOpic) treatment groups ($p>0.05$). No significant different in the proportion of secondary follicles was observed in the control, 1µM, 10µM and 100µM bpV (HOpic) treatment groups ($p>0.05$).

A comparison the ovarian tissue culture in control medium in to those treated with medium containing various concentrations of 740 Y-P displayed that there was

a difference in the distribution of the developmental stages of the ovarian follicle populations (see figure 5.10). The control group had a significantly higher proportion of quiescent follicles at 37.7±1.1% in comparison to those treated with 740 Y-P at 26.6±1.5%, 25.6±1.1%, 22.6±1.5% and 19.3±1.5% in 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P ($p<0.001$). No significant difference was seen in the various concentrations of 740 Y-P ($p>0.05$). The control group had a significantly lower proportion of primary follicles at 56.1±1.0% in comparison to 66.7±0.8%, 68.3±1.0%, 72.1±0.7% and 74.9±1.5% in 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P ($p<0.001$). Again no significant difference was seen in the various concentrations of 740 Y-P ($p>0.05$), although there does appears to be a trend towards a dose-dependent increase in activation with an increase in the concentration of 740 Y-P. No significant difference was observed in the proportion of secondary follicles between the control, 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P treatment groups ($p>0.05$).

Table 5:1 Data collected from the different treatment groups to calculate the distribution of the ovarian follicles

	Treatment	No. Samples	No. Follicles	No. Quiescent Follicles	No. Primary Follicles	No. Secondary Follicles
bpV (HOpic)	Uncultured	24	748	422	312	14
	Control	24	760	276	438	46
	1μM bpV (HOpic)	24	691	180	483	28
	10μM bpV (HOpic)	24	677	176	451	50
	10μM bpV (HOpic)	24	576	153	384	39
740 Y-P	Uncultured	24	713	424	277	12
	Control	24	943	354	530	59
	0.1 μg/ml 740 Y-P	24	716	191	482	43
	1 μg/ml 740 Y-P	24	702	182	481	39
	10 μg/ml 740 Y-P	24	681	149	496	36
	100 μg/ml 740 Y-P	24	680	144	491	45

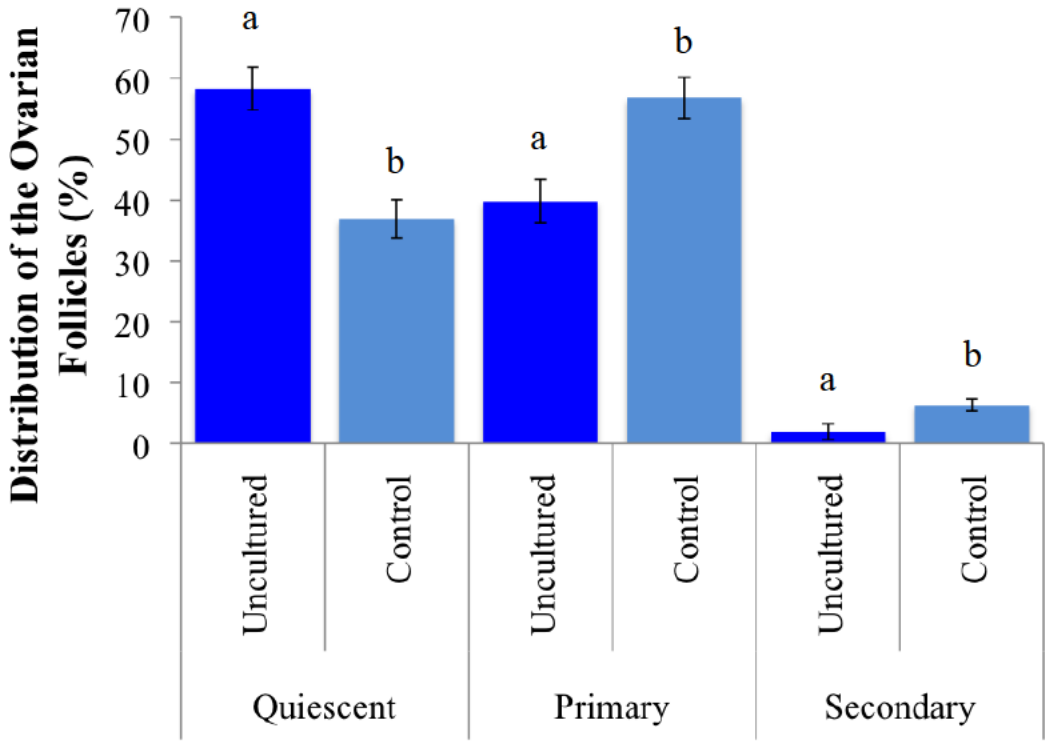


Figure 5.8: Distribution of the Different Developmental Stages of Ovarian Follicles between Uncultured Tissue and Tissue Cultured with Control Medium. The distribution of the ovarian follicles is seen to significantly change between the uncultured tissue and control cultured tissue. The uncultured tissue is seen to have a higher proportion of quiescent follicles and lower proportion of primary and secondary follicles in comparison to the cultured tissue ($p<0.0001$). Means that have different letters are significantly different from one another within each developmental stage ($p<0.0001$). Mean±sem, n=48.

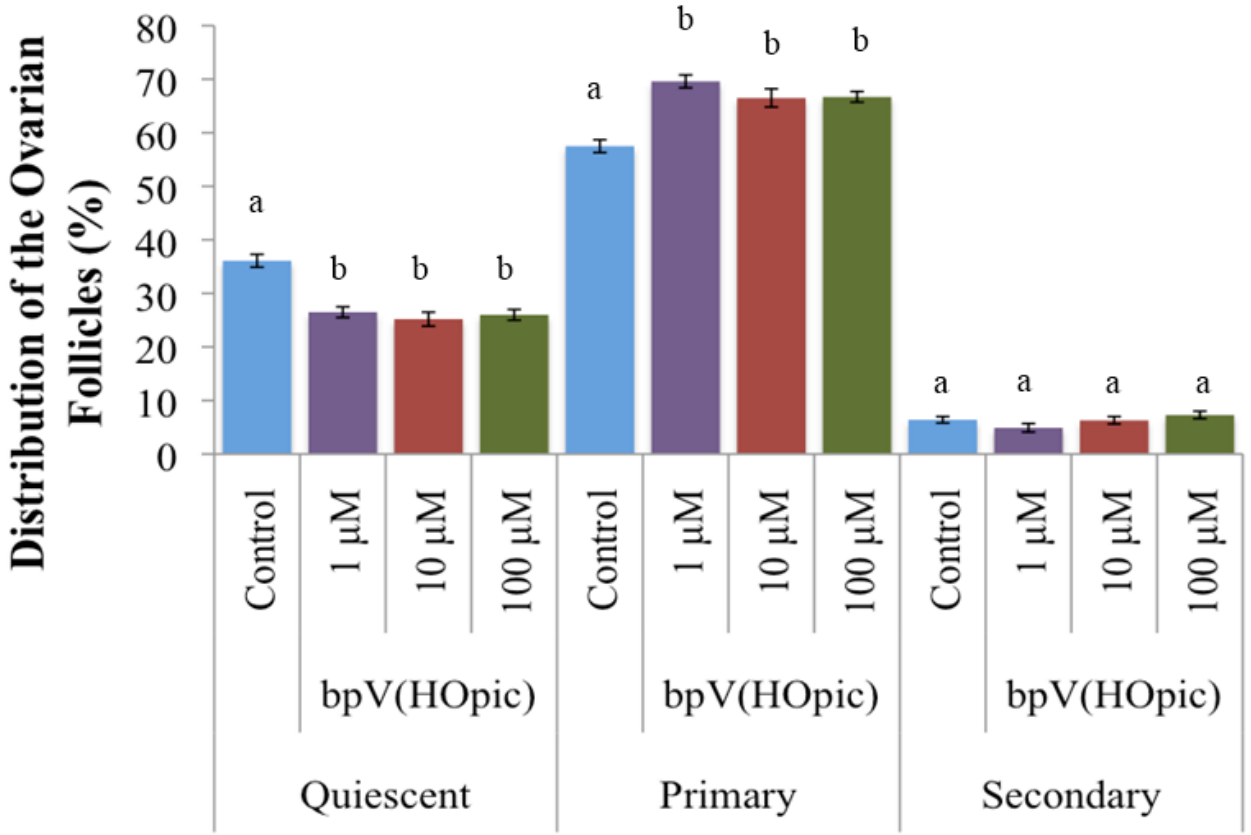


Figure 5.9: Distribution of the Different Developmental Stages of the Ovarian Follicles after Treatment with bpV (HOpic). The distribution of the ovarian follicles is seen to significantly change after treatment with bpV (HOpic) in comparison to the control. The control is seen to have a higher proportion of quiescent follicles and lower proportion of primary follicles in comparison to the tissue treated with the various concentrations of bpV (HOpic) ($p<0.001$). No sigificant difference is observed in the distribution of the quiescent and primary follicles in the different concentrations of bpV (HOpic) ($p>0.05$). No significant difference is seen in the proportion of secondary follicles between any of the treatment groups ($p>0.05$). Means that have different letters are significantly different from one another in each developmental stage ($p<0.001$). Mean±sem, n=24.

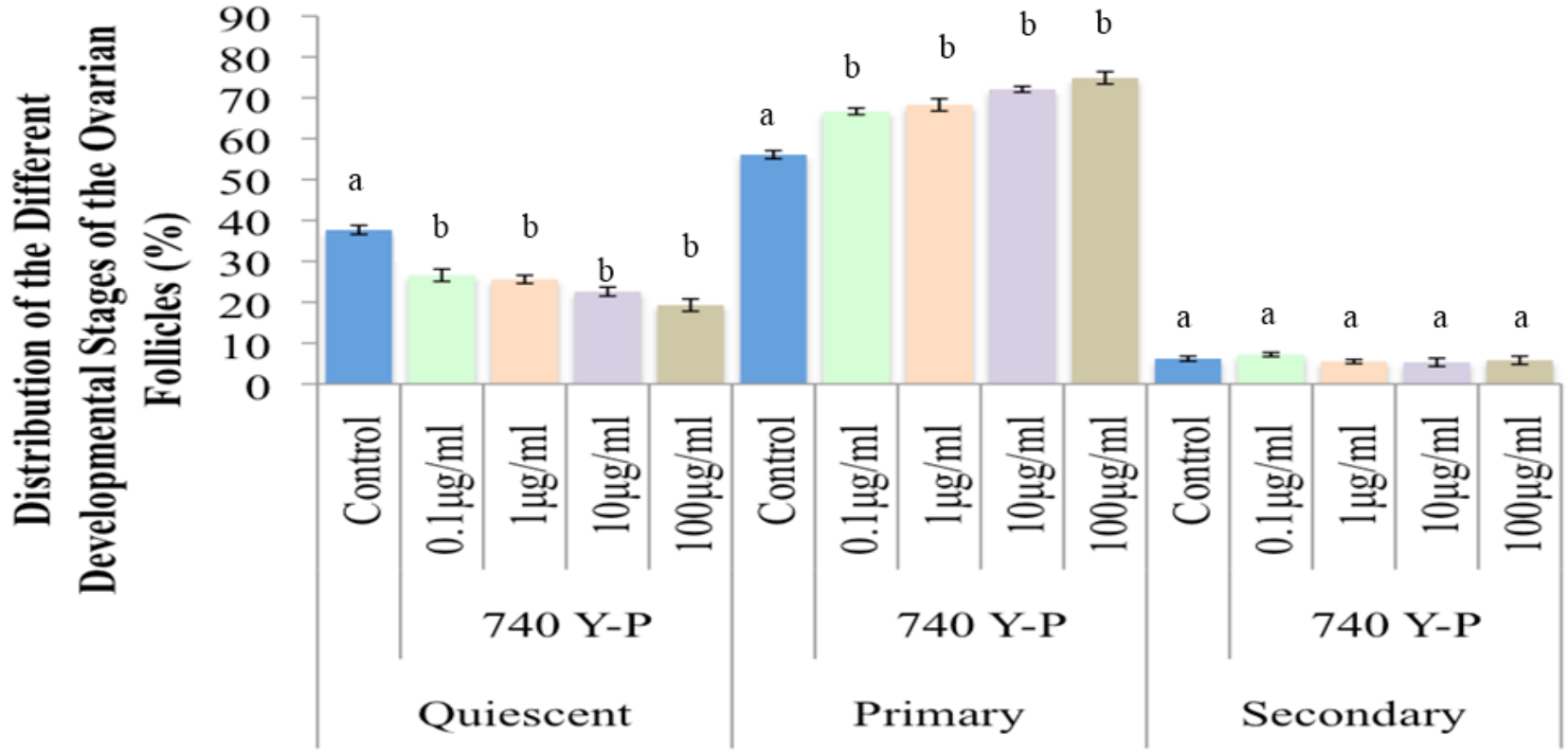


Figure 5.10 Distribution of the Different Developmental Stages of the Ovarian Follicles after Treatment with 740 Y-P. The distribution of the ovarian follicles is seen to significantly change after treatment with 740 Y-P in comparison to the control. The control is seen to have a higher proportion of quiescent follicles and lower proportion of primary follicles in comparison to the tissue treated with the various concentrations of 740 Y-P ($p<0.001$). No sigificant difference is observed in the distribution of the quiescent and primary follicles treated with the different concentrations of 740 Y-P ($p>0.05$). No significant different is seen in the number of secondary follicles between any of the treatment groups ($p>0.05$). Means that have different letters are significantly different from one another within each developmental stage ($p<0.001$). Mean±sem, n=24.

5.3.3 The Health of the Ovarian Follicle Populations and Concentration of the Ovarian Follicles within the Cortical Strips after Treatment with bpV (HOpic) and 740 Y-P

The health of the ovarian follicle populations were examined after treatment with both bpV (HOpic) and 740 Y-P and compared to the control group. Table 5.2 displays a summary of the data collected from the different treatment groups**.** Between the uncultured tissue and tissue cultured with control medium there was seen to be no significant difference in the proportion of healthy of the quiescent, primary or secondary follicles, with 67.9±1.9%, 66.1±1.8% and 60.2±5.9% of the quiescent, primary and secondary follicles seen to be healthy in the uncultured samples compared to 66.2±1.2%, 67.0±1.1% and 57.8±5.0% of the quiescent, primary and secondary follicles seen to be healthy in the control cultured samples ($p>0.05$) (see figure 5.11). No significant difference was observed in the concentration of the ovarian follicles within the cortical strips at 154.34 ±11.4 ovarian follicles per mm^3 in the uncultured samples compared to 160 ±13.8 ovarian follicles per mm^3 in the control cultured samples ($p>0.05$).

The health of the quiescent follicles was impacted by treatments with both bpV (HOpic) and 740 Y-P. Treatment with bpV (HOpic) led to the proportion of healthy quiescent follicles decreasing from 66.4±1.9% in control to 45.2±3.0%, 40.9±2.9%, 14.8±2.2% in 1μM, 10μM and 100μM bpV (HOpic) respectively in a dose-dependent manner ($p<0.05$) (see figure 5.12). After treatment with 740 Y-P there was a decrease in health in the quiescent follicles from 66.0±1.6% in the control to 52.7±2.7%, 49.6±2.3%, 40.1±4.9%, 37.2±3.9% in 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P respectively, with a significant reduction in the health at the two highest concentration of 740 Y-P in comparison to the two lowest concentrations of 740 Y-P ($n=24$) ($p<0.01$) (see figure 5.13).

Similarly, the health of the primary follicle populations decreased after treatment with both bpV (HOpic) and 740 Y-P. With the primary follicle health decreasing from 68.8±1.5% in the control to 27.3±1.5%, 23.5±1.6% and 9.3±1.4% in 1μM, 10μM and 100μM bpV (HOpic) respectively in a dose-dependent manner ($p<0.05$) (see figure 5.14). After treatment with 740 Y-P the health of the primary

follicles decreased from 65.2±1.5% in the control to 41.7±1.2%, 38.5±1.0%, 25.4±1.5% and 23.9±1.3% in 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P respectively, with a further significant decrease in health at the two higher concentrations of 740 Y-P compared to the two lower concentrations of 740 Y-P ($p<0.01$) (see figure 5.15).

The health of the secondary follicle population was also compared between the controls and the various treatment groups of bpV (Hopic) and 740 Y-P. No significant difference was observed in the health of the secondary follicles population between the different treatment groups ($p>0.05$) (see figure 5.16).

No significant difference was observed in the concentration of ovarian follicles in the control group and the tissue treated with the various different concentrations of bpV (HOpic) or 740 Y-P ($p>0.05$) (see figure 5.17).

Table 5.2 Data collected from each treatment group used to calculate follicular health within each population

	Treatment	No. Samples	No Healthy Quiescent	No Unhealthy Quiescent	No. Healthy Primary	No. Unhealthy Primary	No. Healthy Secondary	No. Unhealthy Secondary
bpV (HOpic)	Uncultured	24	294	128	198	114	9	5
	Control	24	182	94	301	137	26	20
	1μM bpV (HOpic)	24	81	99	131	352	16	12
	10μM bpV (HOpic)	24	68	108	106	345	30	21
	10μM bpV (HOpic)	24	24	129	37	347	22	17
740 Y-P	Uncultured	24	283	141	186	91	6	6
	Control	24	236	118	344	186	35	24
	0.1 μg/ml 740 Y-P	24	99	92	198	284	21	22
	1 μg/ml 740 Y-P	24	88	94	185	296	20	19
	10 μg/ml 740 Y-P	24	51	98	125	371	19	17
	100 μg/ml 740 Y-P	24	46	98	118	373	23	22

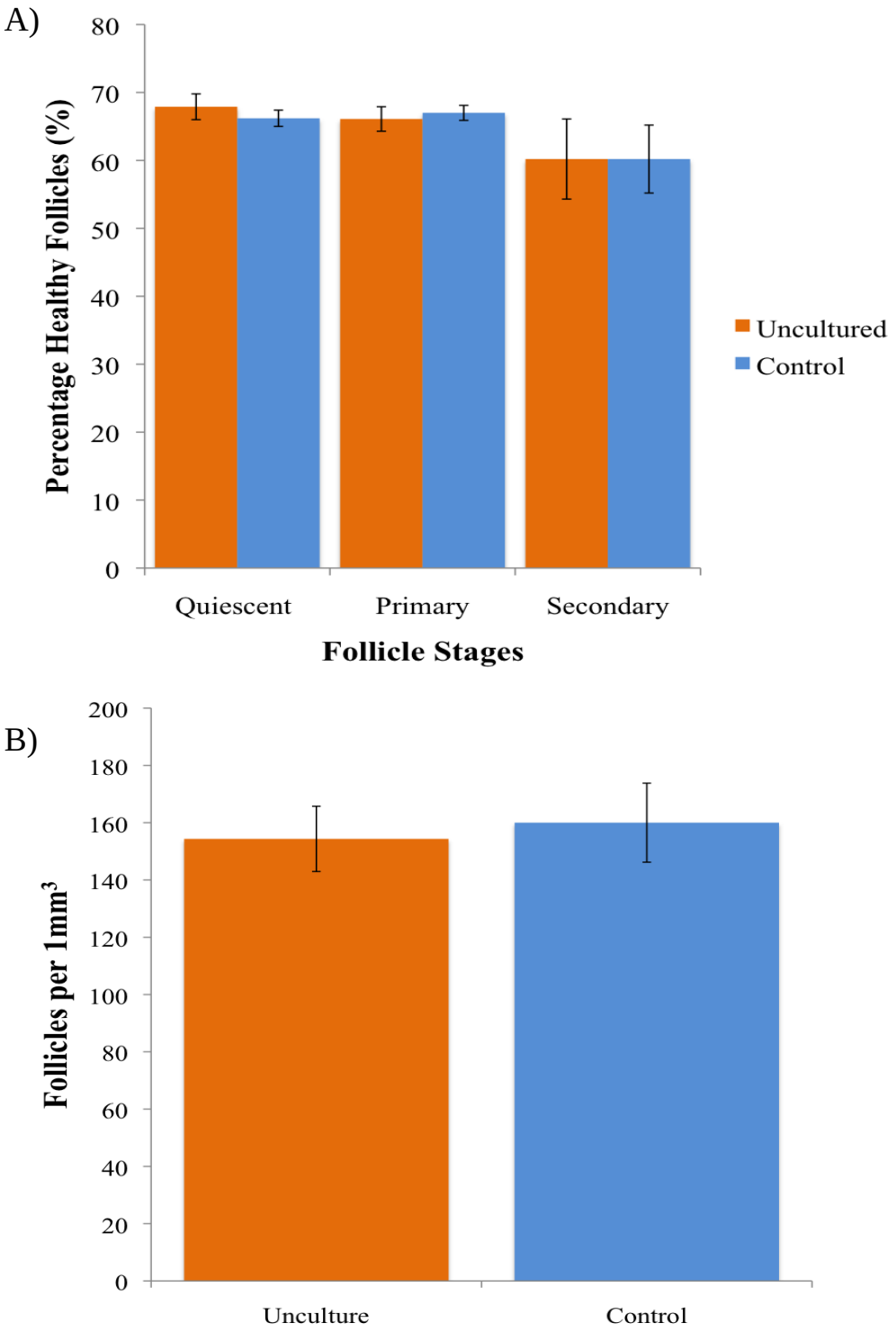


Figure 5.11: A Comparison of Follicular Health and Concentration of Follicles between the Uncultured Tissue Compared with the Control Cultured Tissue. There was no significant decrease in the a) health of the quiescent, primary or secondary follicles in the uncultured tissue in comparison to the control cultured tissue ($p<0.05$). No significant difference was seen in b) the concentration of the ovarian follicles found in the uncultured tissue in comparison to the control ($n=48$) ($p<0.05$).

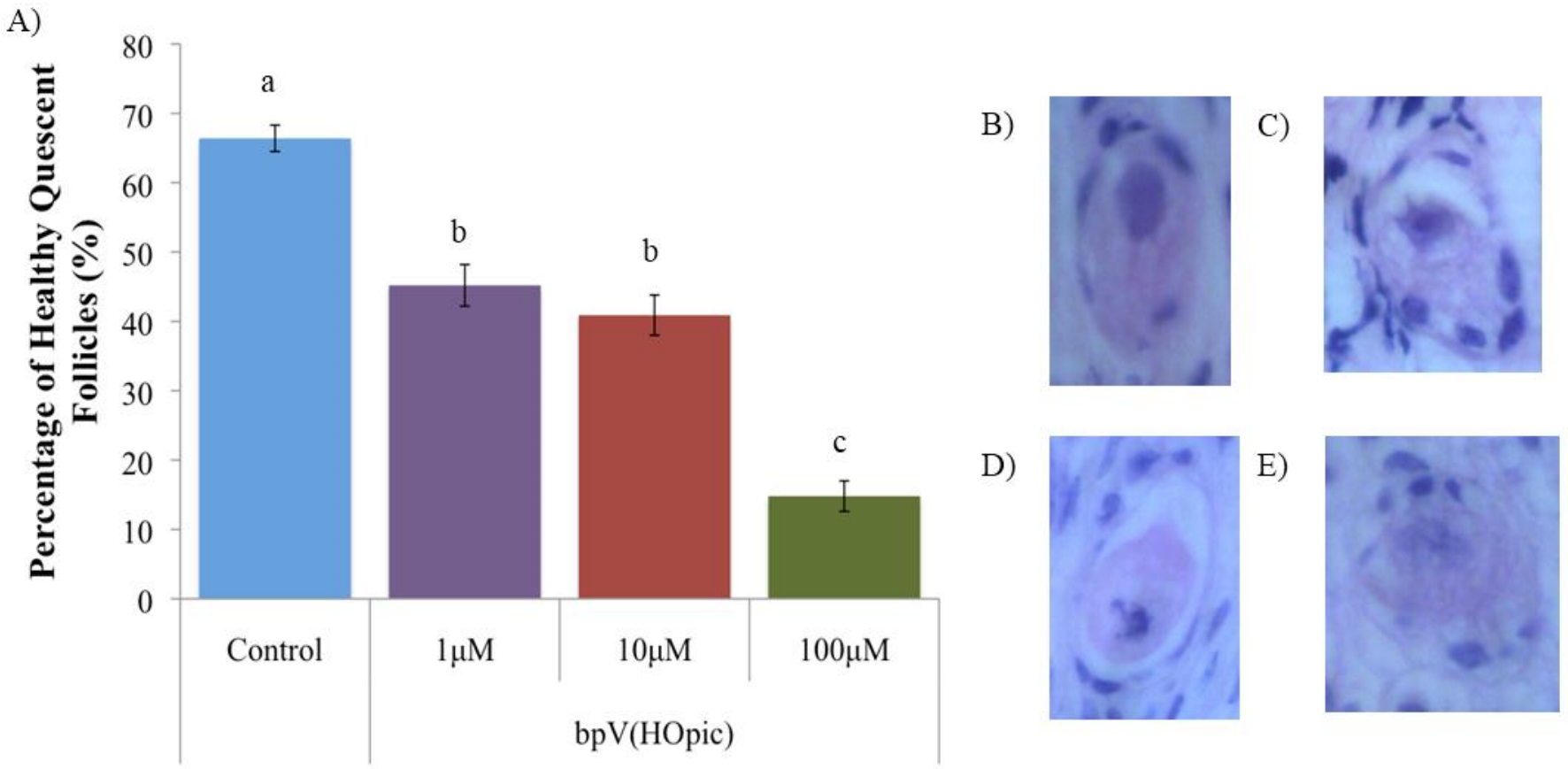


Figure 5.12: Percentage Health of Quiescent Follicle Population after Treatment with bpV (HOpic). There was a significant decrease in the A) health of the quiescent follicles after treatment with bpV (HOpic) in comparison to the control ($p>0.05$). This decrease in health was dose-dependent seen by a decrease in health with an increase in concentration of bpV (HOpic). Means that have different letters are significantly different from one another ($p>0.05$). Mean±sem, n=24. Image B) displays a quiescent follicle found in the control compared to C) 1µM bpV (HOpic), D) 10µM bpV (HOpic) and E) 100µM bpV (HOpic).

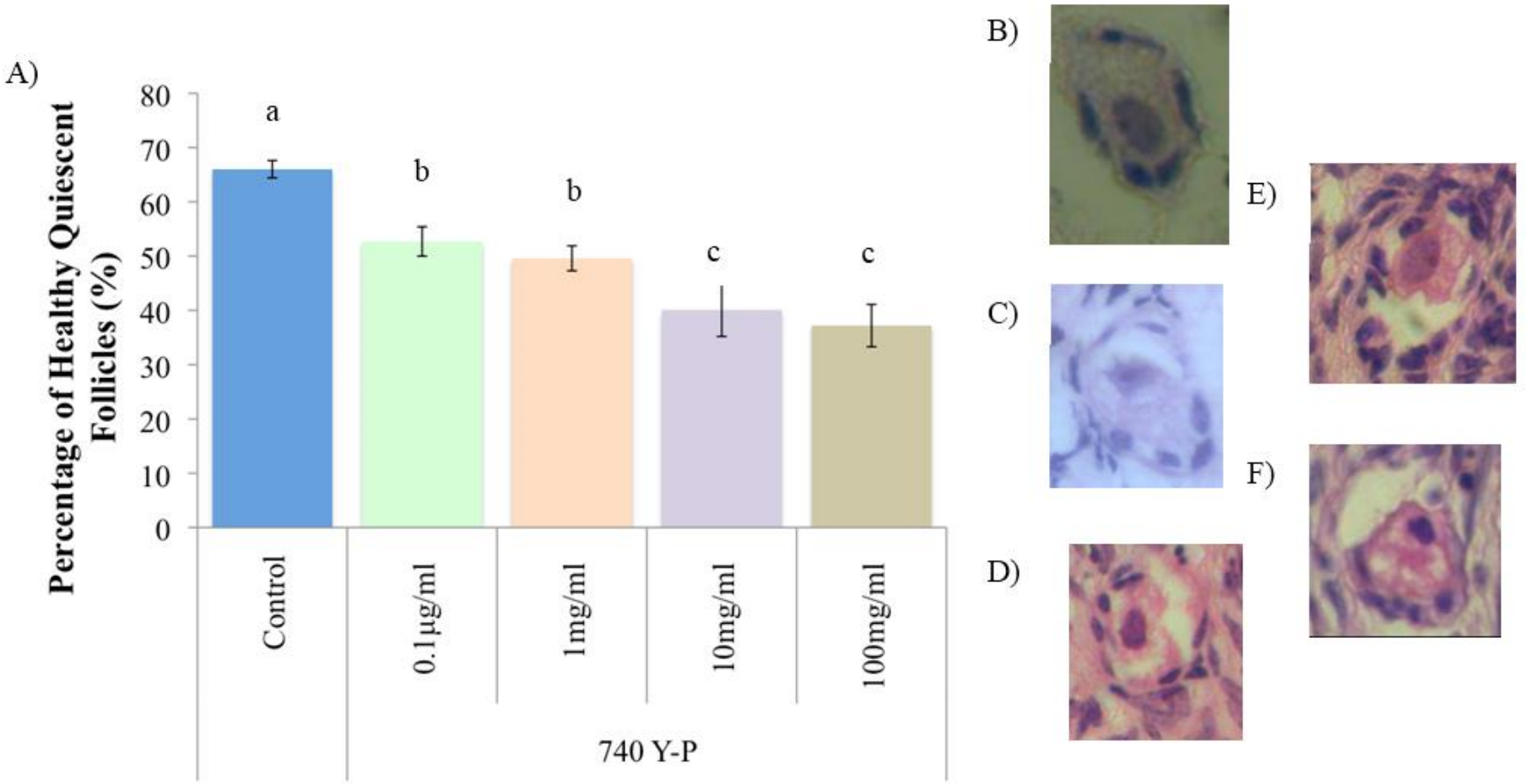


Figure 5.13: Percentage Health of Quiescent Follicle Population after Treatment with 740 Y-P. There was a significant decrease in the A) health of the quiescent follicles after treatment with 740 Y-P in comparison to the control ($p<0.05$). This decrease in health is larger in the highest two concentrations of 740 Y-P in comparison to the two lower concentrations ($p<0.05$). Means that have different letters are significantly different from one another ($p<0.05$). Mean±sem, n=24. Image B) displays a quiescent follicle found in the control compared to C) 0.1µg/ml 740 Y-P, D) 1 µg/ml 740 Y-P, E) 10 µg/ml 740 Y-P and F) 100 µg/ml 740 Y-P.

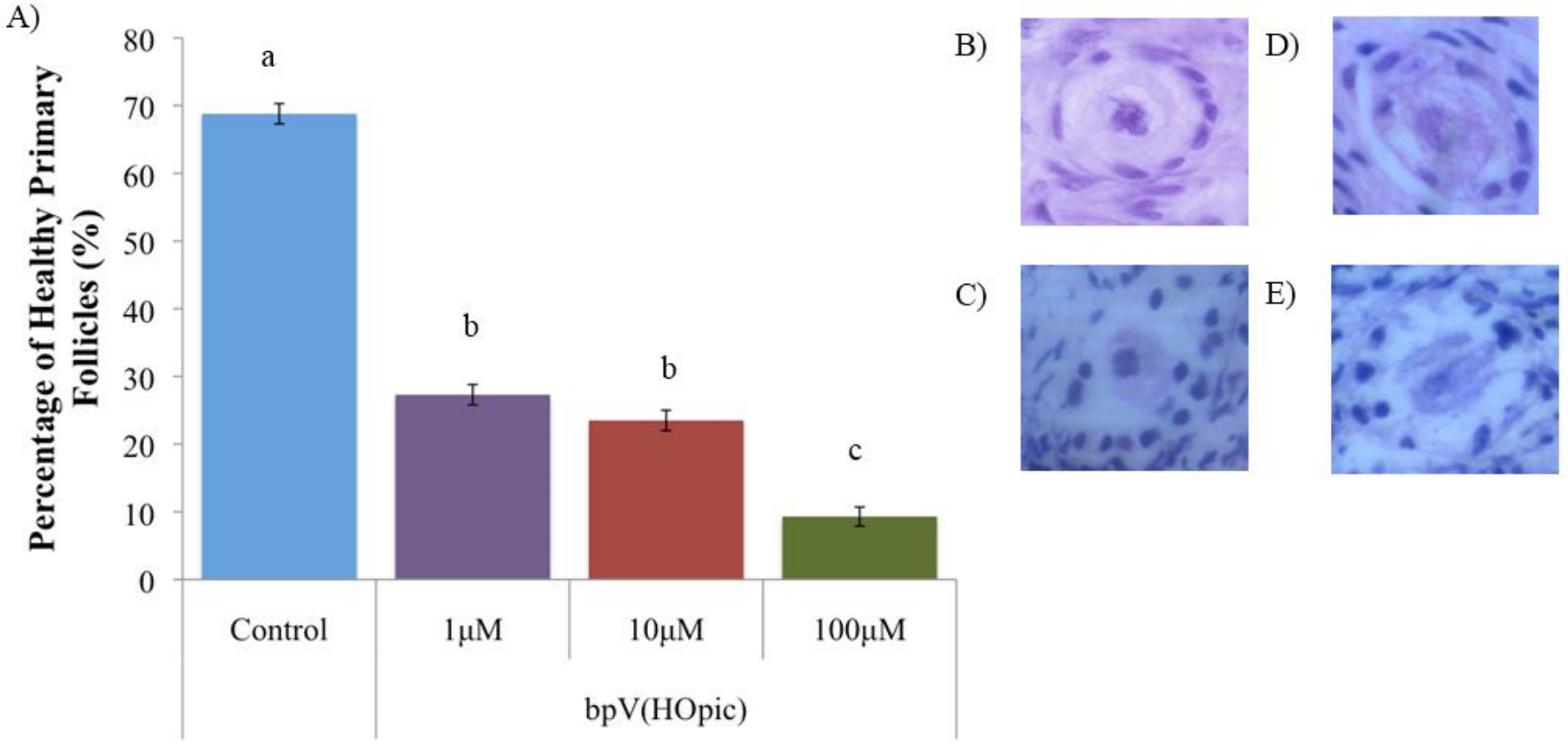


Figure 5.14: Percentage Health of Primary Follicle Population after Treatment with bpV (HOpic). There was a significant decrease in the A) health of the primary follicles after treatment with bpV (HOpic) in comparison to the control. This decrease in health is dose-dependent seen by a decrease in health with an increase in concentration ($p<0.05$). Means that have different letters are significantly different from one another ($p<0.05$). Mean±sem, n=24. Image B) displays a primary follicle found in the control compared to C) 1µM bpV (HOpic), D) 10µM bpV (HOpic) and E) 100µM bpV (HOpic).

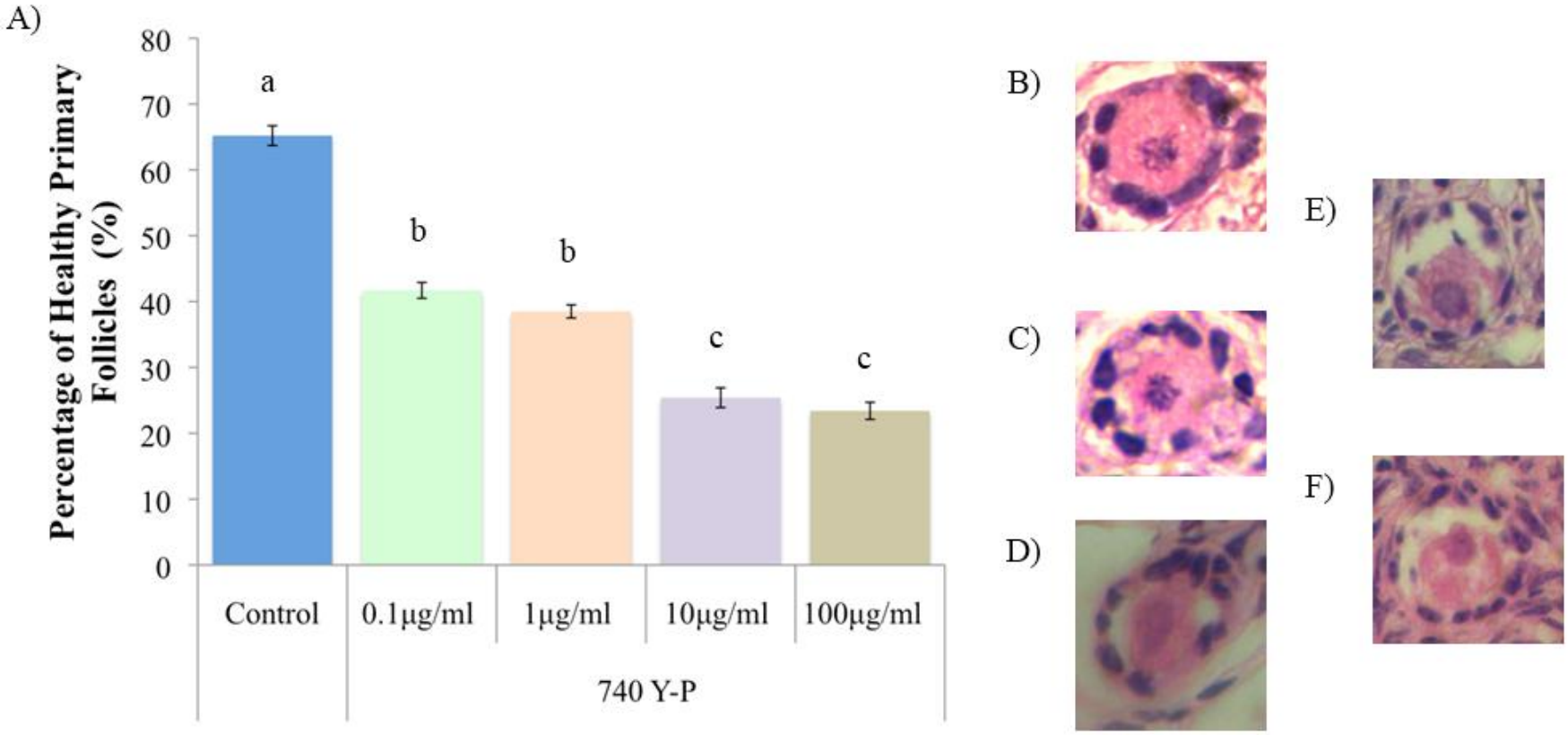


Figure 5.15: Percentage Health of Primary Follicle Population after Treatment with 740 Y-P. There was a significant decrease in the A) health of the quiescent follicles after treatment with 740 Y-P in comparison to the control (n=24). This decrease in health is larger in the highest two concentrations of 740 Y-P in comparison to the two lower concentrations ($p<0.05$). Means that have different letters are significantly different from one another ($p<0.05$). Mean±sem, n=24. Image B) displays a quiescent follicle found in the control compared to C) 0.1µg/ml 740 Y-P, D) 1 µg/ml 740 Y-P, E) 10 µg/ml 740 Y-P and F) 100 µg/ml 740 Y-P.

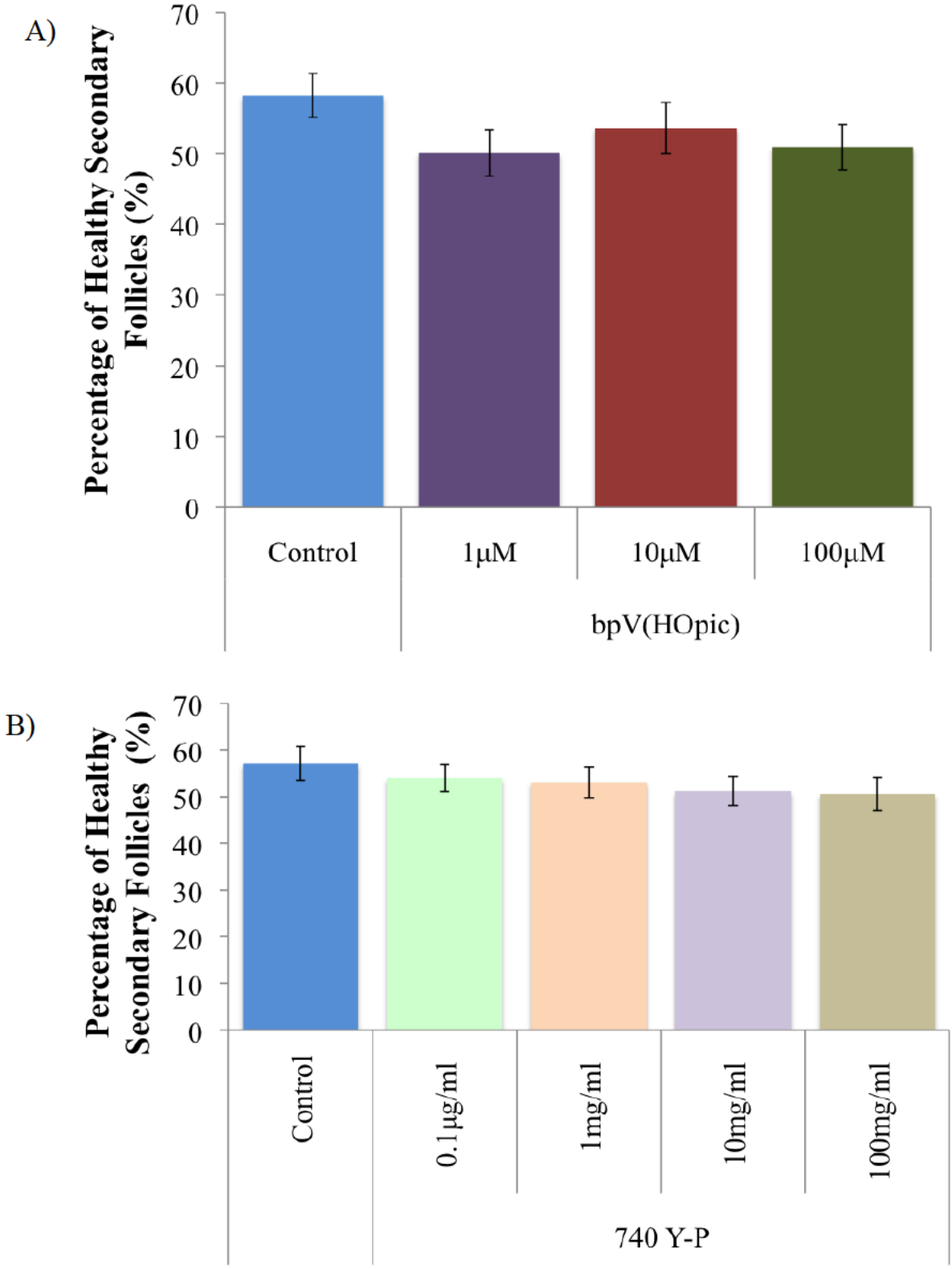


Figure: 5.16 Health of the Secondary Follicles after Treatment with bpV(HOpic) and 740 Y-P. There was seen to be no significant difference in the health of the secondary follicles after treatment with A) bpV (HOpic) ($p>0.05$) or B) 740 Y-P ($p>0.05$). Mean±sem, n=24.

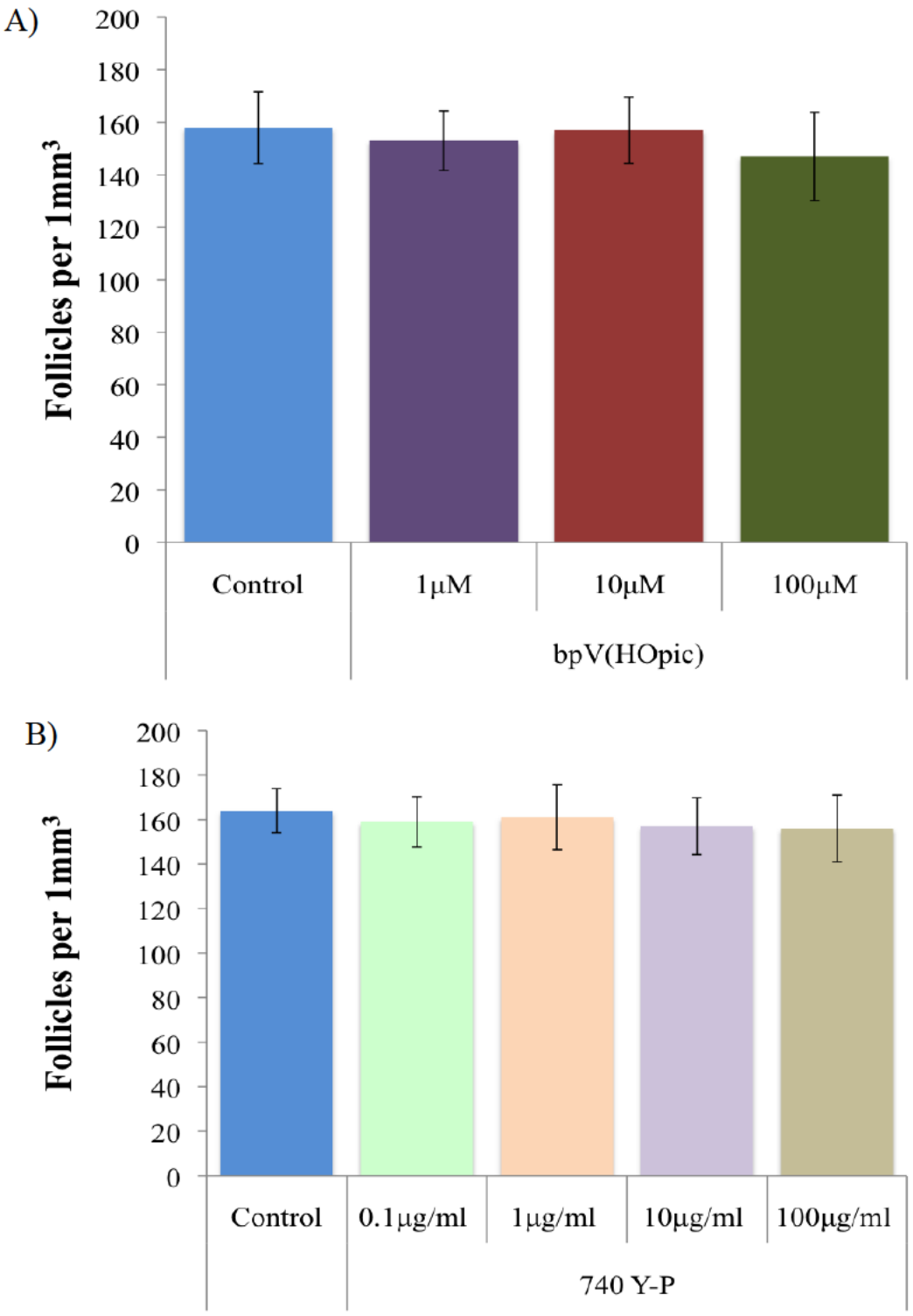


Figure 5.17: Concentration of Ovarain Follicles after Culture. No significant difference was observed in the concentration of the ovarian follilces after treatment with A) bpV (HOpic) ($p>0.05$) or B) 740 Y-P ($p>0.05$). Mean±sem, n=24.

5.3.4 Follicle and Oocyte Diameters after Treatment with bpV (HOpic) and 740 Y-P

No significant difference was observed in the size of the quiescent follicles or their oocyte between the uncultured (n= 846) and control cultured group (n=630) ($p>0.05$). However, tissue treated with bpV (HOpic) had an increase in the diameter of the quiescent follicles to a mean diameter of 26.1±0.2μm, 28.9±0.3μm and 27.5±0.2μm in 1μM (n=180), 10μM (n=176) and 100μM (n=153) bpV (HOpic) compared to 24.0±0.1μm in the control (n=276) ($p<0.001$) (see figure 5.18 and 5.19). The oocytes within the quiescent follicles also increased in diameter from 20.7±0.1μm in the control (n=276) to 21.6±0.2μm, 24.1±0.2μm and 22.8±0.2μm in 1μM (n=180), 10μM (n=176) and 100μM (n=153) bpV (HOpic) ($p<0.001$) (see figure 5.18 and 5.19).

Similarly, the diameters of the quiescent follicles increased after treatment with 740 Y-P. The quiescent follicles had a mean diameter of 23.9±0.2μm in the control (n=354) whereas, those treated with 740 Y-P had a mean diameter of 26.6±0.2μm, 28.4±0.2μm, 31.8±0.3μm and 29.6±0.3μm in 0.1μg/ml (n=191), 1μg/ml (n=182), 10μg/ml (n=149) and 100μg/ml (n=144) 740 Y-P ($p<0.001$) (see figure 5.18 and 5.18). The oocytes within the quiescent follicles had a mean diameter of 22.2±0.2μm, 23.8±0.2μm, 27.5±0.3μm and 25.7±0.2μm in 0.1μg/ml (n=191), 1μg/ml (n=182), 10μg/ml (n=149) and 100μg/ml (n=144) 740 Y-P in comparison to 20.5±0.2μm in the control (n=354) ($p<0.001$) (see figure 5.18 and 5.19).

No signifcant difference was observed in the size of the primary follicles or their oocytes between the uncultured (n=589) and control cultured ovarian tissue (n=968) ($p>0.05$). However, treatment with bpV (HOpic) resulted in an increase in the mean primary follicle diameter in comparison to the control (n=438) at 28.9±0.1μm in diameter compared to 30.4±0.1μm, 32.2±0.1μm and 30.8±0.1μm in 1μM (n=483), 10μM (n=451) and 100μM (n=384) bpV (HOpic) respectively ($p<0.001$) (see figure 5.20 and 5.21).

The oocytes within the primary follicles increased from a mean diameter of 23.5±0.1μm in the control (n=438) to 24.8±0.1μm, 26.3±0.1μm and 25.1±0.1μm in 1μM (n=483), 10μM (n=451) and 100μM (n=384) bpV (HOpic) ($p<0.001$) (see

figure 5.20 and 5.21). Treatment with 740 Y-P also caused an increase in the mean diameter of the primary follicles with the control (n=530) at 28.6±0.1μm in comparison to 31.6±0.1μm, 34.0±0.1μm, 34.7±0.1μm and 34.6 ±0.1μm in 0.1μg/ml (n=482), 1μg/ml (n=481), 10μg/ml (n=496) and 100μg/ml (n=491) 740 Y-P ($p<0.001$) (see figure 5.20 and 5.21). The oocytes within the primary follicles had a mean diameter of 22.2±0.2μm, 23.8±0.2μm, 27.5±0.3μm and 25.7±0.2μm in 0.1μg/ml (n=482), 1μg/ml (n=481), 10μg/ml (n=496) and 100μg/ml (n=491) 740 Y-P in comparison to 20.5±0.2μm in the control (n=530) ($p<0.001$) (see figure 5.20 and 5.21).

No significant difference was observed in the diameter of the secondary follicles or their oocytes between the uncultured (n=26) and control cultured ovarian tissue (n=105). No significant difference was observed in the diameter of secondary follicles or their oocytes after treatment with control (n=46), 1μM (n=28), 10μM (n=50) or 100μM (n=39) bpV (HOpic) ($p>0.05$) (see figure 5.22). No significant difference was observed in the diameter of secondary follicles or their oocytes after treatment with control (n=59), 0.1μg/ml (n=43), 1μg/ml (n=39), 10μg/ml (n=36) or 100μg/ml (n=45) 740 Y-P ($p>0.05$).

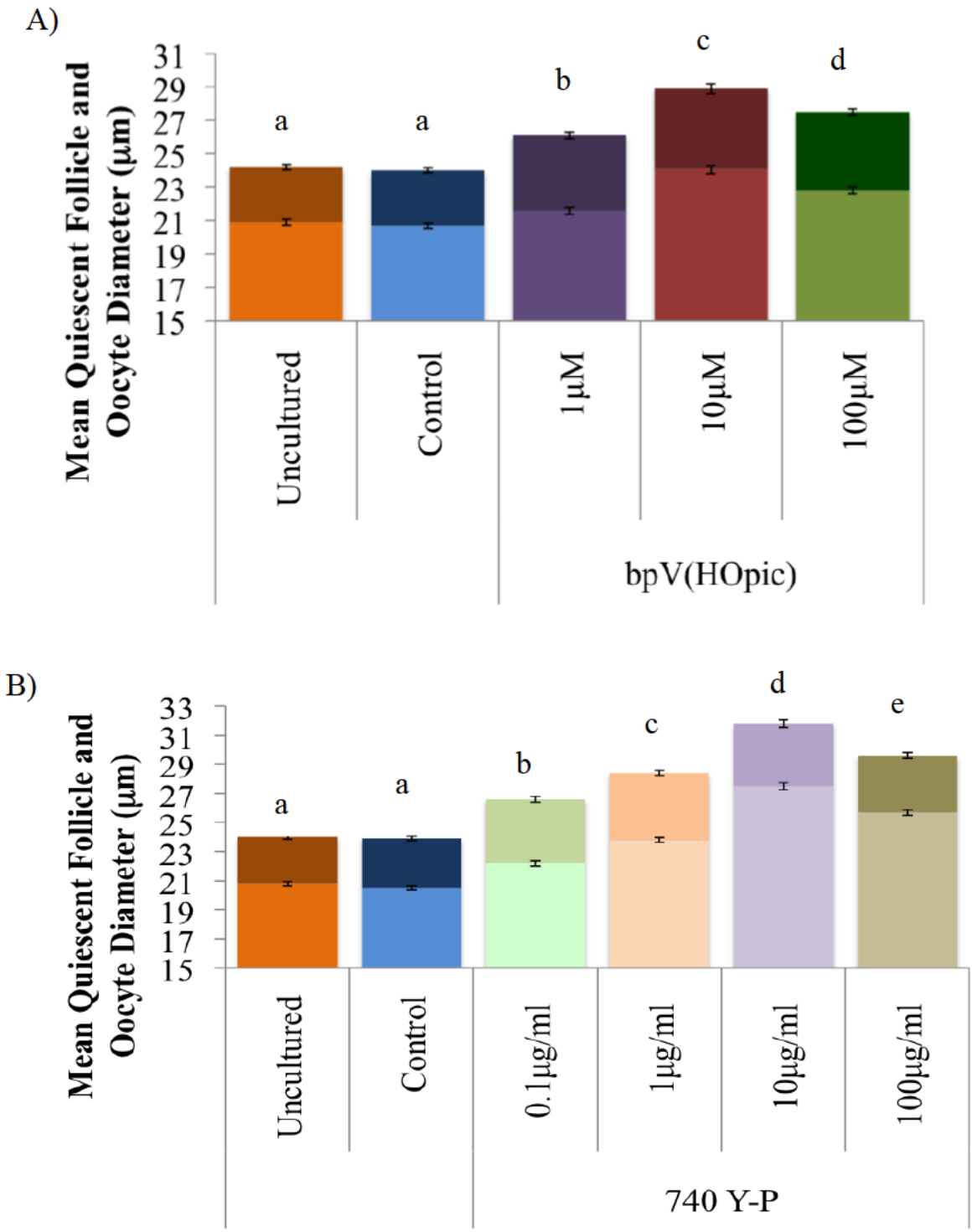
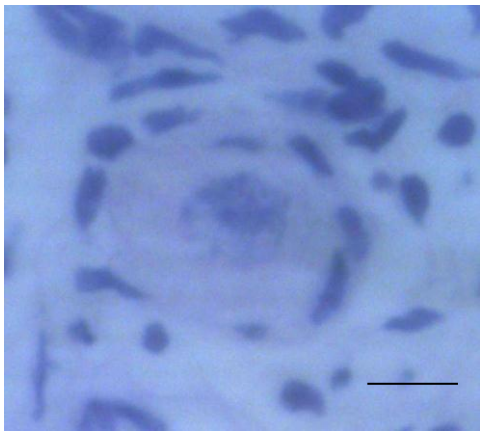
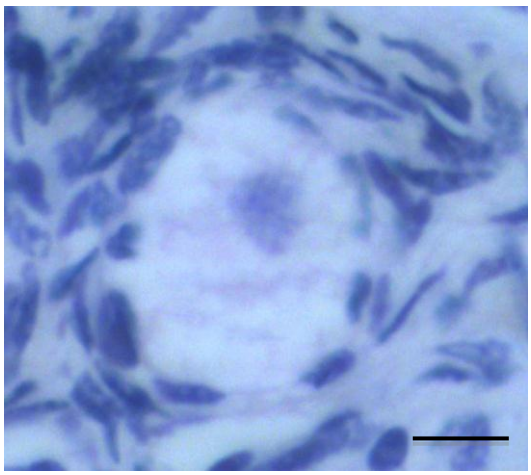


Figure 5.18: Mean Diameter of the Quiescent Follicles and their Oocytes. These graphs display the mean diameter of the quiescent follicles and their respective oocytes after treatment with A) bpV (HOpic) or B) 740 Y-P in comparison to the control. The darker colours represent the mean follicle diameter and the ligher colour represents the mean oocyte diameter within each treatment group. Treatment with both bpV (HOpic) and 740 Y-P caused a significant increase in the diameter of the oocyte and the diameter of the oocytes ($p<0.001$). Means that have different letters are significantly different from one another ($p<0.001$), mean±sem.

A)



B)



C)

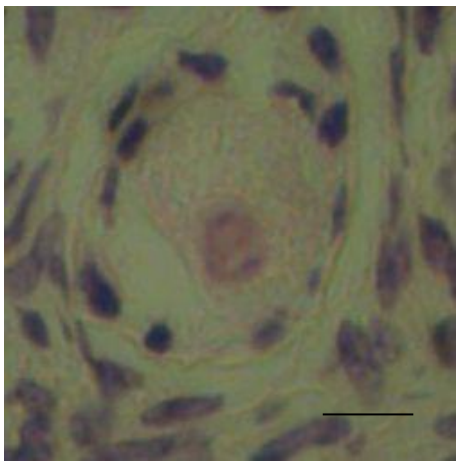


Figure 5.19: Images of Quiescent Follicles after Treatment with bpV (HOpic) and 740 Y-P. Images show the change in size of the quiescent follicles after treatment with 740 Y-P or bpV (HOpic). It is possible to see the A) quiescent follicles from the control group is smaller in comparison to those treated with B) bpV (HOpic) (1μM) and C) 740 Y-P (0.1μg/ml) ($p<0.05$). Scale bar is equal to 10μm.

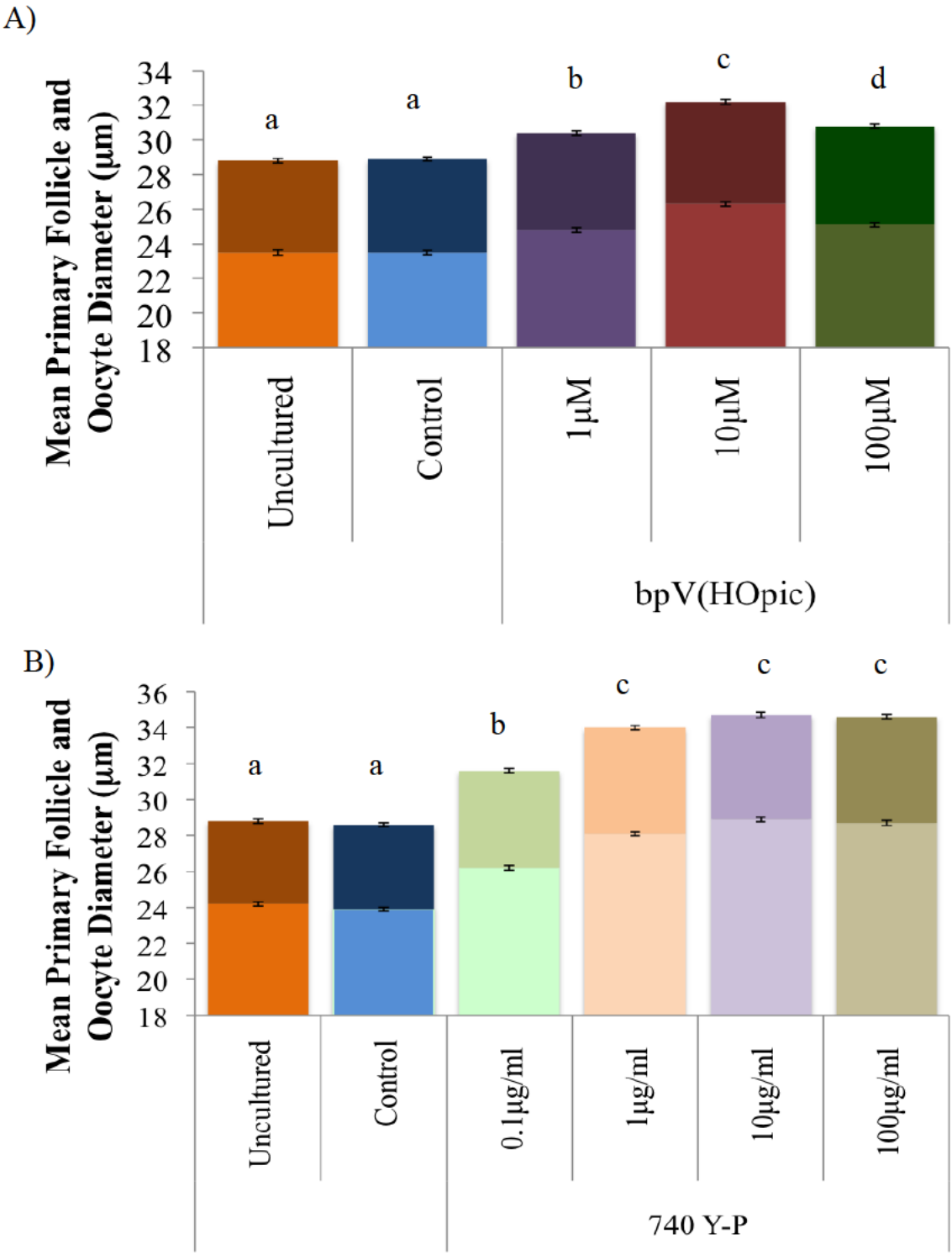


Figure 5.20: Mean Diameter of the Primary Follicles and their Oocytes. These graphs display the mean diameter of the primary follicles and their oocytes after treatment with A) bpV (HOpic) or B) 740 Y-P in comparison to the control. The darker colours represent the mean follicle diameter and the ligher colour represents the mean oocyte diameter within each treatment group. Treatment with both bpV (HOpic) and 740 Y-P caused a significant increase in the diameter of the primary follicles and the diameter of their oocytes ($p>0.001$). Means that have different letters are significantly different from one another ($p>0.001$), mean±sem.

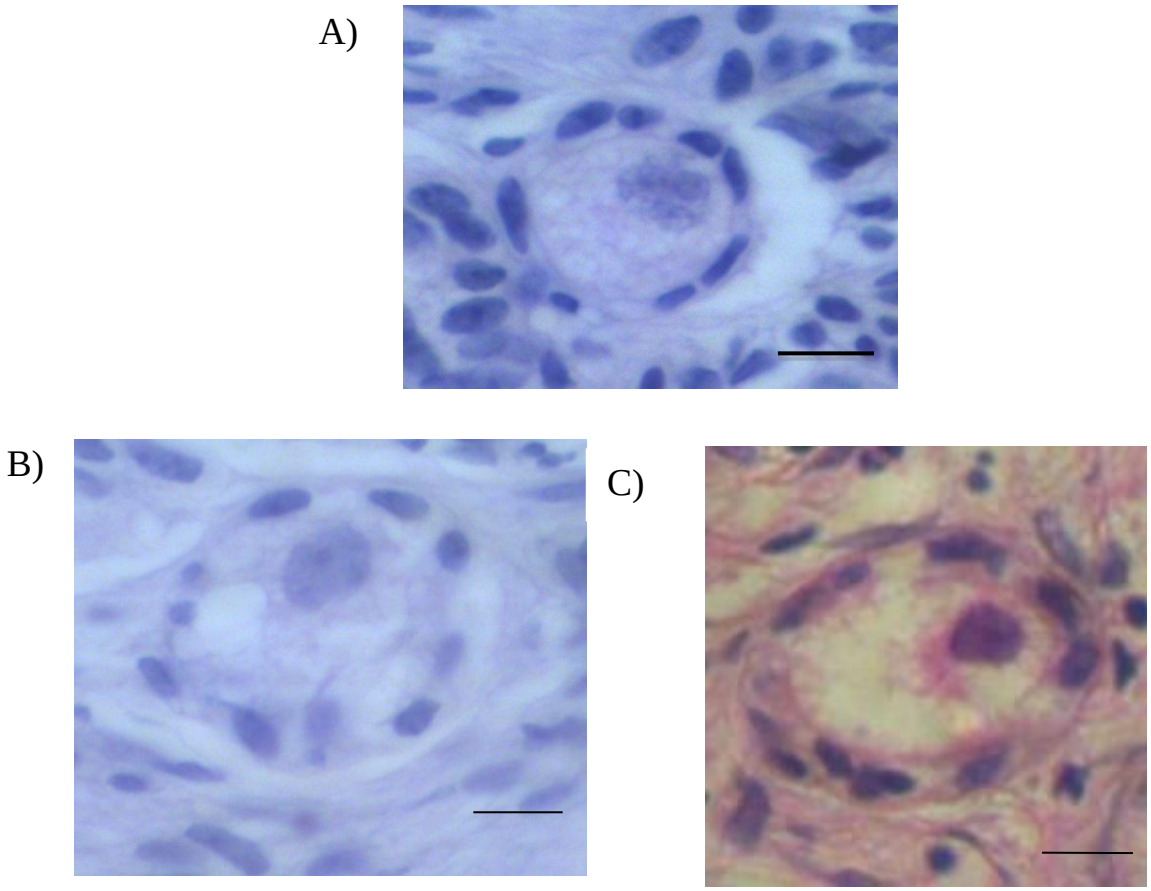


Figure 5.21: Images of Primary Follicles after Treatment with bpV (HOpic) and 740 Y-P. Images display the enlargement of the primary follicles after treatment with bpV (HOpic) or 740 Y-P in comparison to the control. The a) control primary follicles are smaller in diameter than the b) bpV (HOpic) (1μM) treated primary follicles ($p<0.05$) and c) 740 Y-P (0.1μg/ml) primary treated follicles ($p<0.05$). Scale bar is equal to 14μm.

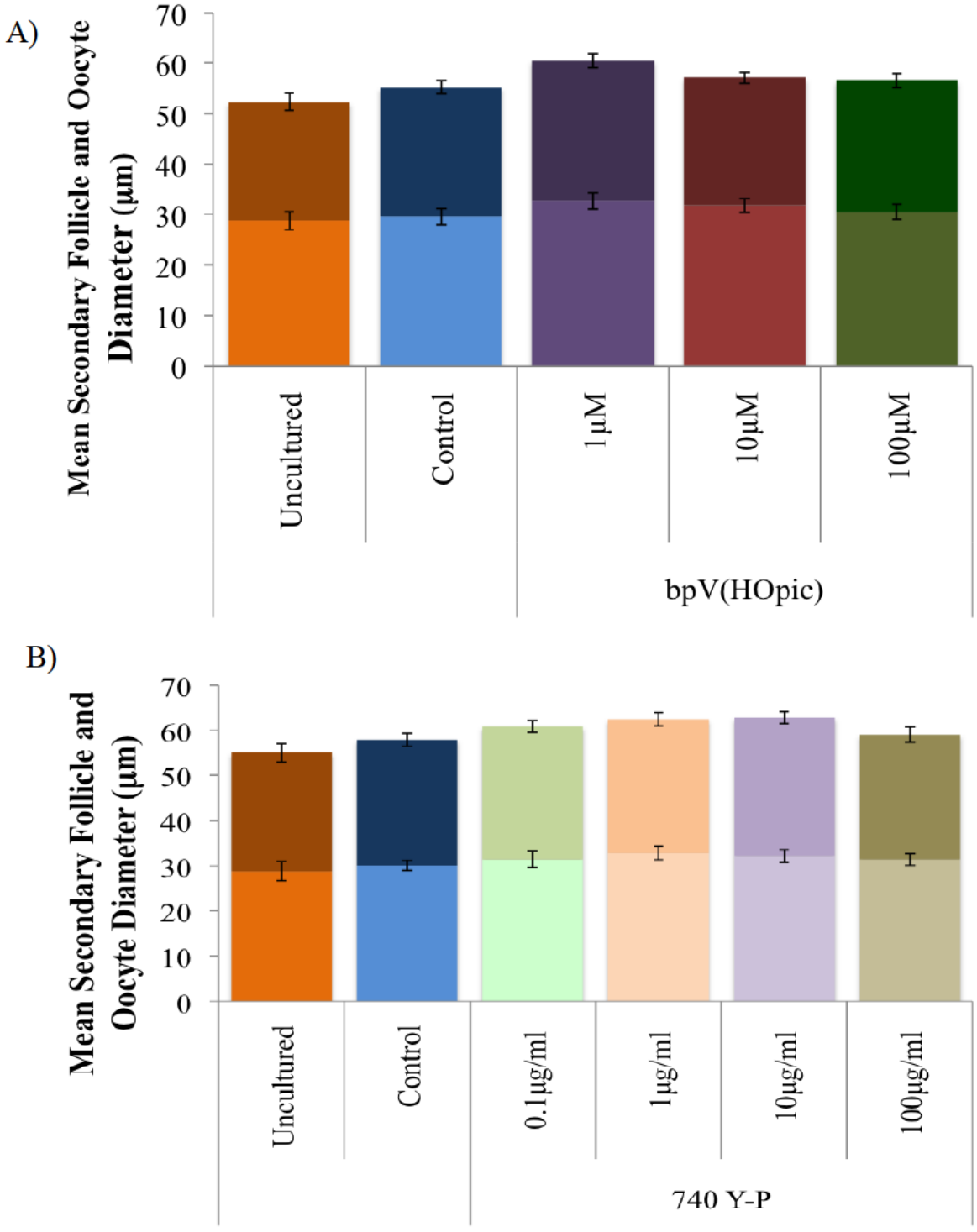


Figure 5.22 Mean Diameter of the Secondary Follicles and their Oocytes. These graphs display the mean diameter of the secondary follicles and their oocytes after treatment with A) bpV (HOpic) or B) 740 Y-P in comparison to the control. The darker colours represent the mean follicle diameter and the ligher colour represents the mean oocyte within each treatment. Neither treatment with bpV (HOpic) or 740 Y-P caused a significant change in the diameter of the secondary follicles or their oocytes ($p>0.05$), mean±sem.

5.3.5 Examining the Diameters of the Healthy and Unhealthy Follicle Populations after Treatment with bpV (HOpic) and 740 Y-P

The diameter of the quiescent and primordial follicles was increased after treatment with both bpV (HOpic) and 740 Y-P, however, there was also a reduction in the proportion of healthy ovarian follicles within the cortical strips. The uncultured samples and control samples displayed no significant difference in the diameters of quiescent or primary follicles. The quiescent follicles within these two groups ranged from 20.25μm to 30μm in diameter, no significant difference was observed between the range of diameters of the healthy and unhealthy quiescent follicles within these two treatment groups. To explore if the change in the diameter of the quiescent follicles after treatment with bpV (HOpic) or 740 Y-P impacted the proportion of the healthy quiescent follicles, the quiescent follicles were divided into those within the control follicle diameter range of 20.25μm to 30μm and compared to those above this range. After treatment with bpV (HOpic) a significantly larger proportion of the healthy quiescent follicles were found within the control diameter range of 20.25μm to 30μm at 72.5±3.1%, 73.7±2.9% and 75.8±3.5% in 1μM, 10μM and 100μM bpV (HOpic) ($p<0.05$) (see figure 5.23). Similarly, after treatment with 740 Y-P a significantly larger proportion of the healthy quiescent follicles were found within the diameter range of 20.25μm to 30μm at 68.8±3.6%, 69.3±3.7%, 73.6±3.5% and 73.9±3.9% in 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P respectively ($p<0.05$).

Within the uncultured and control samples the primary follicles ranged from 25.5μm to 37μm, no significant difference was observed between the diameters of the healthy and unhealthy primary follicle populations within these two treatment groups. To explore if the change in the diameter of the primary follicles impacted their health, the proportion of the healthy primary follicles were seperated into those within the control primary follicle diameter range of 25.5μm to 37μm compared to those above this range. After treatment with bpV (HOpic) a significantly larger proportion of the healthy primary follicle population was found within the control diameter range of 20.25μm to 30μm at 74.5±3.4%, 75.7±3.9% and 72.8±3.3% in 1μM, 10μM and 100μM bpV (HOpic) ($n=24$) ($p<0.05$) (see figure 5.24). Similarly,

after treatment with 740 Y-P a significantly larger proportion of the healthy primary follicles were found within the control diameter range of 20.25μm to 30μm at 72.8±3.6%, 69.9±3.5%, 75.6±3.1% and 73.9±3.6% in 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P respectively (n=24) ($p<0.05$).

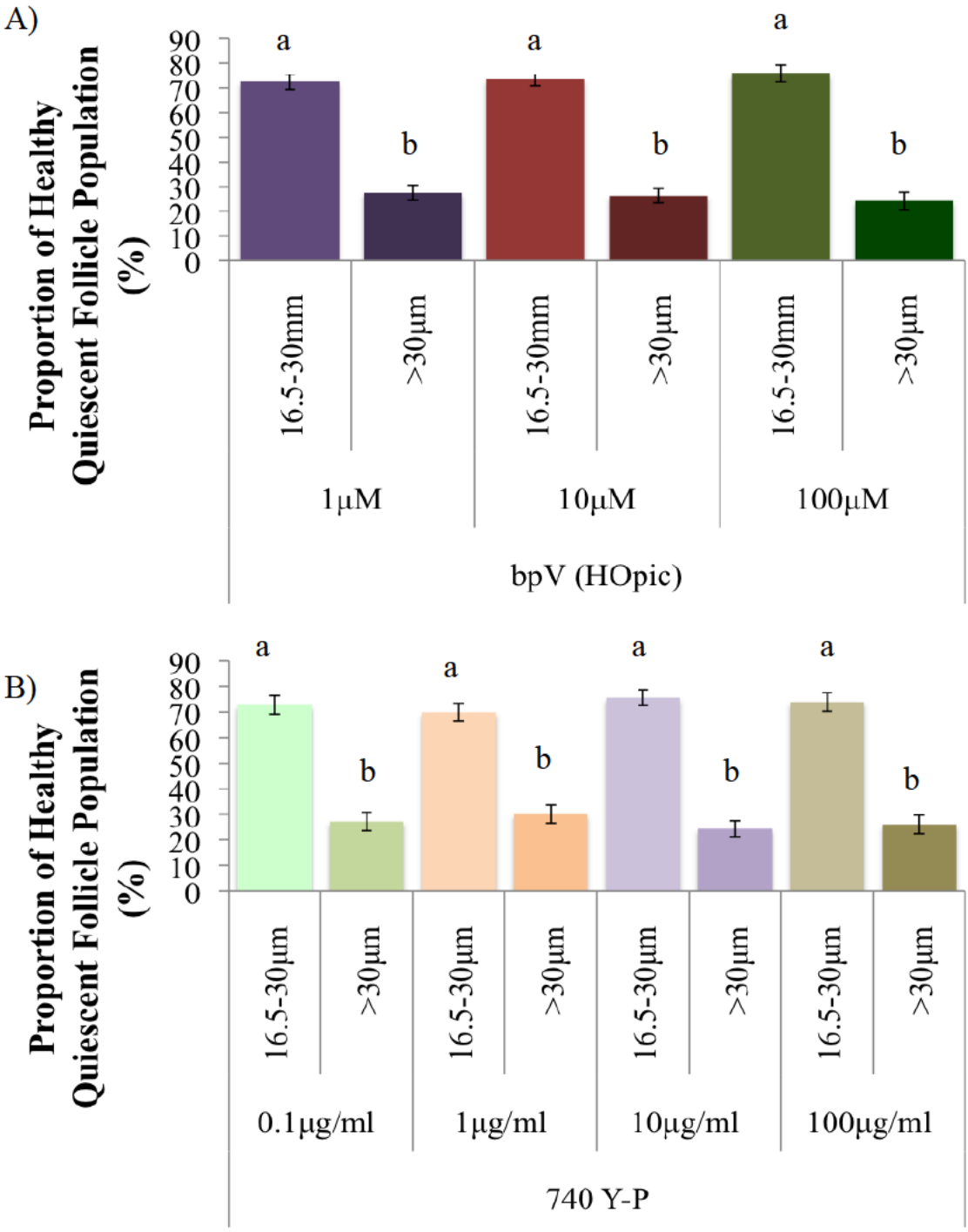


Figure 5.23 Proportion of the Healthy Quiescent Follicles Population in the Control Follicle Size Range. These graphs display the proportion of the healthy quiescent follicle population in the control follicles size range of 16.5-30µm compared to those above it after treatment with A) bpV (HOpic) and B) 740 Y-P. A significantly larger proportion of the healthy quiescent follicle population is within the control follicles size range of 16.5-30µm ($p<0.05$). Means that have different letters are significantly different from one another ($p>0.001$), mean±sem. The quiescent follicle population above 30µm in the various bpV (HOpic) and 740 Y-P concentrations is approximately 17-24% of the overall follicle population.

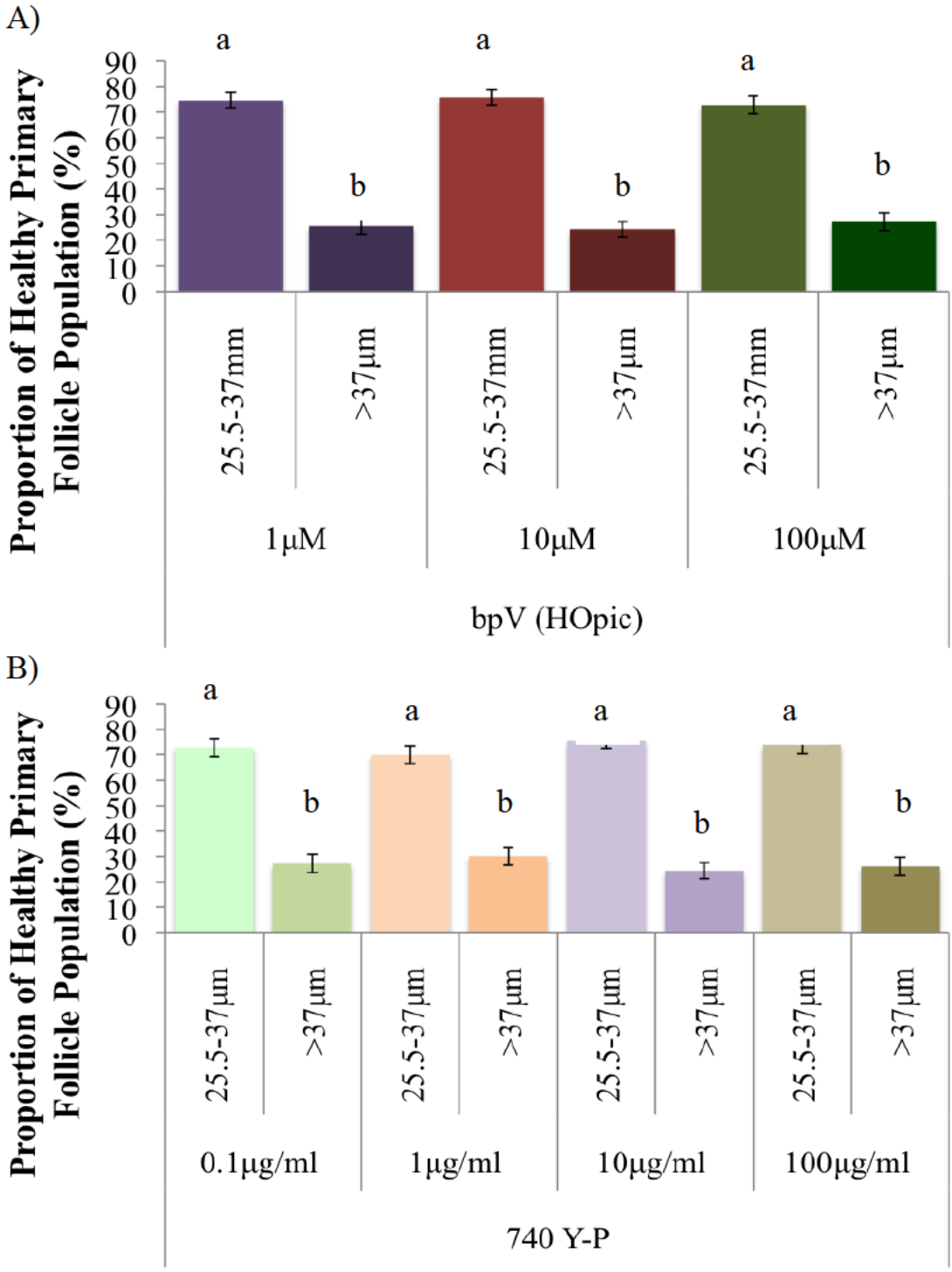


Figure 5.24 Proportion of the Healthy Primary Follicles Population in the Control Follicle Size Range. These graphs display the proportion of the healthy primary follicle population in the control follicles size range of 25.5-37µm compared to those above it after treatment with A) bpV (HOpic) and B) 740 Y-P. A significantly larger proportion of the healthy primary follicle population is within the control follicles size range of 25.5-37µm ($p<0.05$). Means that have different letters are significantly different from one another ($p>0.001$), mean±sem. The primary follicle population above 37µm in the various bpV (HOpic) and 740 Y-P concentrations is approximately 19-31% of the overall follicle population.

5.4 Discussion

This study aimed to futher explore the role of the PI3K pathway in a large mono-ovulate species. To achieve this the two pharmological compounds bpV(HOpic) and 740 Y-P were utilised within an *in vitro* bovine model. When the PI3K pathway is stimulated, PI3K activates Akt through phosphorylation to mediate the downstream components of the pathway (Blume-Jensen and Hunter, 2001; Brunet et al., 2001; Cantley, 2002; Engelman et al., 2006; Stokoe, 2005; Vanderhyden, 2002). Within the *in vitro* bovine model there was shown to be an increase in the levels of p-Akt after bovine ovarian tissue fragments were treated with either bpV(HOpic) or 740 Y-P therefore, indicating both cause an up-regulation of the PI3K pathway. The data obtained within this study is comparible to previous studies as the up-regulation of the PI3K pathway in the bovine caused an increase in the activation of quiescent follicles similar to that observed in the knockout mouse models studies (Reddy et al., 2008, John et al., 2008, Castrillon et al., 2003, Rajareddy et al., 2007) and the mouse (Li et al. 2010) and the human (Li et al. 2010, McLaughlin et al., 2014) studies using these pharmacological compounds. Although, there was an increase in the proportion of activated primoridal follicles and therefore, an increase in the proportion of primary follicles the portion of secondary follicles was not increased after treatment with either bpv (HOpic) or 740 Y-P in comparison to the control.

In the previous study by Li et al., 2010 in the mouse model used a combination of 740 Y-P and bpV (HOpic) which resulted in an increase in the level of follicular activation compared to just bpV (HOpic) alone (Li et al, 2010) although, no significant change was seen between the combination of bpV (HOpic) and 740 Y-P in comprison to 740 Y-P alone (Li et al., 2010). To examined if the level of PI3K stimulation impacted the level of activation of the primordial follicles in this study a range of concentrations of bpV (HOpic) or 740 Y-P were utilised, which has not been done in any previous study in the mouse or human. This study conveyed that different concentration of the both pharmological compounds did not impacted the percentage of quiescent follicles activated. Although, the various concentrations of 740Y-P did display a non-significant trend towards an increase in activation with an

increase in the concentration of 740 Y-P, further investigation using a wider range of concentrations is required to confirm this trend.

Alongside the increase in primordial follicle activation in the bovine model, there was a decrease in follicular health in both the quiescent and primary follicle populations. The health of the quiescent follicles was not explored in the mouse models (Reddy et al., 2008, John et al., 2008, Castrillon et al., 2003, Rajareddy et al., 2007, Li et al., 2010). The studies using the human model did explore the health of the follicle population and it was observed that there was a decrease in the health of larger isolated follicles after the initial activation of the quiescent follicles via the PI3K pathway (McLaughlin et al., 2014). Therefore, this study is the first to observe that these pharmacological compounds can impact not only the growing follicle population but also the quiescent follicle population. The level of health of the quiescent and primary follicle populations was reduced after treament with both bpV (HOpic) and 740 Y-P. The different concentrations of bpV (HOpic) and 740 Y-P displayed that the higher concentration of both compounds were more detrimental to the health of both the quiescent and primary follicle populations. Despite 740Y-P causing a greater increase in follicle activation it appears to have a less detrimental impact on the health of the quiescent and primary follicles in comparison to bpV (HOpic). This could be due to the different ways in which these pharmacological compounds stimulate the PI3K pathway. bpV (HOpic) is a PTEN inhibitor and causes an up-regulation of the PI3K pathway by binding to PTEN to prevent PIP3 being converted back into PIP2 (Bevan et al., 1995; Posner et al., 1994; Schmidt et al., 2004). Whereas, 740 Y-P is a PI3K activator that up-regulates the PI3K pathway by binding to the p85 subunit of PI3K inhibiting its inhibition on p110, so p110 is in its high activate state (Derossi et al., 1998; Li et al., 2010). When Pten was knockout out in the granulosa cells there was a reduction in the number of apoptotic granulosa cells, but it did not cause an increase in the activation of the quiescent follicle population. Granulosa cells only contain a low level of Pten protein (Fan et al., 2008), and as bpV (HOpic) relies on the amount of Pten present this could prevent bpV (HOpic) stimulating the granulosa cell alongside the oocyte. Whereas, 740 Y-P might be able stimulate the PI3K pathway in the granulosa cells as it stimulates PI3K

itself, which could cause a reduction in the apoptotic granulosa cells in comparison to those treated bpV (HOpic) leading to healthier quiescent and primary follicles.

The decrease in the health of the primary follicles created via stimulation of the PI3K pathway is likely to be a key factor in there being no increase in the proportion of secondary follicles was observed as the quality of an ovarian follicle and its oocyte is a key factor in determining if the ovarian follicle will undergo atresia or continue through folliculogenesis (Blondin and Sirard, 1995; Hendriksen et al., 2004; Salamone et al., 1999). Therefore, the increase in degenerative primary follicles will mean that many of them are unable to progress to the secondary stage of follicular development. Alternatively, the secondary follicle may have required more time to develop from the primary follicle population as follicular development is known to be a protracted process (Adams and Pierson, 1995) and perhaps if left longer more secondary follicles would develop therefore, further investigation is required to better understand why the increase in primordial follicle activation does not result in an increase in the number of secondary follicles.

The stimulation of the PI3K pathway way also caused an increase in the mean diameter of the quiescent and primary follicles. This increase in the diameter of the quiescent and primary follicles appears to be due to an enlargement of the oocytes within the follicle. Enlarged oocytes in the primary and quiescent follicles have been observed in previous mouse studies although not quantified (Castrillon et al., 2003; John et al., 2008; Reddy et al., 2008). This study shows that the increase in mean diameters of the quiescent and primary follicles is significant as well as showing similarities between the mouse and the bovine models. This is the first study to show that the PI3K pathway can cause this increase in oocyte diameter in a large mono-ovulate species, as this has not been observed in the previous human studies (Li et al., 2010, McLaughlin et al., 2014). The diameter of an oocyte is usually within a set range depending upon developmental stage that the ovarian follicles are within as described in the study Fair., 2003. In this study treatment with either bpV (HOpic) or 740 Y-P resulted in the oocytes within the quiescent follicle population being similar in diameter to oocytes found in the primary follicles in the control group. Similarly, the primary follicles oocytes diameters were comparable to some of the oocytes within the secondary follicles within the control group. This enlargement of the

oocytes within the ovarian follicles suggests that there has been a disruption in the co-ordinated growth between an oocyte and its granulosa cells. The co-ordinated growth of an oocyte and its granulosa cells is essential in maintaining the health of an ovarian follicle (Cecconi et al., 2004; Hutt et al., 2006; Otsuka and Shimasaki, 2002; Wu et al., 2004) and a disruption within this could be a contributing factor to the poor health of the quiescent and primary follicles observed within this study. The PI3K pathway is thought to control the follicular activation via the oocyte as oocyte-specific deletion of PTEN (Reddy et al., 2008) caused an increase in follicular activation, whereas granulosa cell-specific deletion of PTEN (Fan et al., 2008) had no impact on follicular activation. The lack of co-ordination after activation of the quiescent follicles through the PI3K pathway could be due to the PI3K pathway only driving the activation of the oocytes, but not the granulosa cells. However, further investigation is required to truly understand this disruption in the co-ordination growth of the oocyte and it's granulosa cells.

In summary manipulating the PI3K pathway causes an up-regulation of the activation of the primary follicles. However, up-regulating the PI3K pathway resulted in a dose-dependent decrease in the health of both the quiescent and primary follicle populations with increasing concentrations of both bpV (HOpic) and 740 Y-P. The PI3K pathway also impacted the regulation of ovarian follicle growth with the oocyte being enlarged in both the quiescent and primary follicle populations. This indicates a disruption in the communication between the oocyte and the granulosa cells although further investigation is required to better understand this.

It has been proposed that these two pharmacological compounds are used in fertility treatments. A live human birth has recently been reported using bpV (HOpic) and 740 Y-P followed by replacement and IVF (Kawamura et al., 2013). However, the study did not have a control and the ovary was cut up which could have an impact on other pathways as well as the PI3K pathway so it is hard to determine if this was the result of the up–regulation of the PI3K pathway. The results in the present study displayed damage to the quiescent and primary follicles can be caused by these pharmacological compounds, indicating that we should be cautious as these results highlight these compounds have the potential to impact the health of not only the growing follicle populations, but also the quiescent follicle population. This

study shows that there was no increase in follicular activation despite an increase in concentration of the two compounds although, there is a decrease in the health with an increase in concentration of both therefore, it might be possible to find a concentration that causes the up-regulation of the PI3K pathway without the negative impact on the health of the quiescent and primary follicles. Further research is required to establish non-detrimental concentrations that can be safely used in clinical applications.

The secondary follicle population had no increase in the population, health or follicle diameter despite the up-regulation of the PI3K pathway. However, the number of quiescent and primary follicles is very much higher than the number of secondary follicles in all treatment groups therefore, it is difficult to see the impact of the PI3K pathway on this later stage of development. To better understand if and how the later stages of follicle development are influence it would be beneficial to continue culturing the secondary population using the second part of the two-step culture system developed by the Telfer laboratory group (McLaughlin and Telfer, 2010; Telfer et al., 2008). This will determine if there is an increase in the number of large secondary follicles produced and the number of secondary follicles could then be further cultured to give a better indication of whether the number of mature oocytes obtained would be increased after increased primordial follicle activation via the PI3K pathway.

Chapter Six:
Exploring the Impact of Increased Primordial Follicle Activation via the PI3K Pathway on Subsequent Follicular Development.

6.1 Introduction

The PI3K pathway has been shown to be important in primordial follicle activation, with the up-regulation of the PI3K pathway causing increased primordial follicle activation as observed in chapter 5 and previous studies in the mouse and human (Castrillon et al., 2003; John et al., 2008; Li et al., 2010; Liu et al., 2007; McLaughlin et al., 2014; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008). Activation of the primordial follicles to become primary follicles is only the first step in the process of folliculogenesis, and is though to be the only step in which the PI3K pathway plays a role (Castrillon et al., 2003; John et al., 2008) (see figure 6.1). However, activation is irreversible, so once a primordial follicle has been activated the primordial follicle must continue through the progressive stages of folliculogenesis or be lost through atresia (Elvin and Matzuk, 1998; Scaramuzzi et al., 2011; Webb and Campbell, 2007; Webb et al., 2004). Although the PI3K pathway does not play a role in the subsequent steps of folliculogenesis, the increase in primordial follicles activation via the stimulation of the PI3K pathway does cause an increase in the number of primary follicles. Therefore, by up-regulating the PI3K pathway and increasing the number of primordial follicles that are activated will there be an increase in the number of mature oocytes produced?

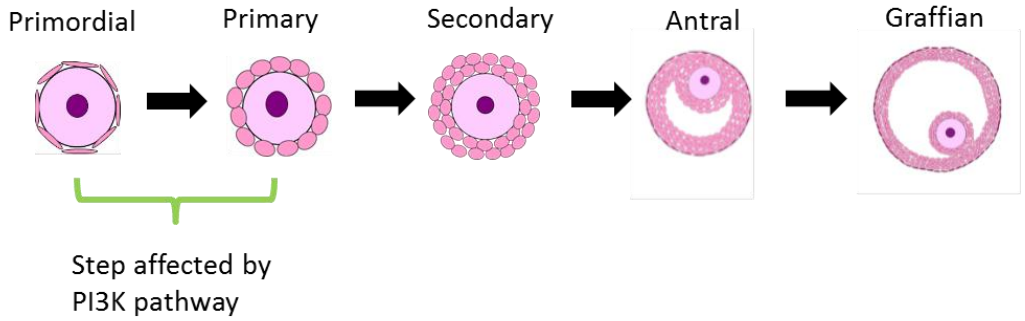


Figure 6.1: Folliculogenesis and the PI3K Pathway. The image displays the various stages of folliculogenesis needed for a mature oocyte to be produced from a primordial follicle. The PI3K pathway is only thought to play a role in the first stage of folliculogenesis as the primordial follicles are activated to become primary follicles.

In chapter 5, activation of the quiescent follicles via the up-regulation of the PI3K pathway resulted in a decrease in the proportion of health primary follicles and altered primary follicles development. In the McLaughlin et al., 2014 study the up-regulation of the PI3K pathway in the human resulted in a decrease in the survival of the secondary follicles (McLaughlin et al., 2014) therefore, it is important to further explore if activation via the PI3K pathway impacts the later stages of folliculogenesis.

To do this the quiescent follicle population was activated using bpV (HOpic) and 740 Y-P, as in chapter 5. After culturing the cortical strips the large secondary follicles were isolated and further cultured to observe the growth and number of large secondary follicles produced after increase follicular activation via the PI3K pathway. The *in vitro* culture system used in this study was developed by Telfer et al., 2008 and McLaughlin and Telfer, 2010, in this system the quiescent follicles activated in the cortical strips must develop to reach a diameter of 100μm before being isolated for the second step of the culture system. Only these follicles selected to be cultured further and eventually reach a stage where their oocytes could be extracted for maturation (McLaughlin and Telfer, 2010; Telfer et al., 2008). The number of large follicles obtained after treatment with bpV (HOpic) or 740 Y-P will therefore, indicate if the up-regulation of the PI3K pathway has the potential to increase the number of fertilisable mature oocytes produced. This chapter also aimed to examine the development and health of each isolated secondary follicles during their culture to explore the impact of up-regulating the PI3K pathway on the later stages of follicular development.

6.2 Materials and Methods

6.2.1 Bovine Tissue Culture

Bovine ovaries were collected from a local abattoir from animals aged between 10-14 months and transported to the laboratory in supplemented M199 medium. Strips of the cortex were removed from the ovaries to a depth of 1-2mm (see figure 6.2). The strips were examined under a light microscope in bovine Leibovitz medium to ensure that all the ovarian follicles within them were less than 40µm in diameter. These cortical strips were further dissected to approximately 0.5-1 mm^2 in size. The cortical strips were treated with bovine McCoy's or bovine McCoy's with 1µM, 10µM or 100µM of bpV (HOpic) or 0.1µg/ml, 1µg/ml, 10µg/ml or 100µg/ml of 740 Y-P in a humidified incubator at 37°C with 5% CO_2 for 24 hours. After 24 hours all the medium was removed and replaced with bovine McCoy's medium only for a further 6 days, with a medium change every 48 hours (as described in chapter 2).

The cortical strips were then placed in bovine Leibovitz medium and examined under a light microscope with a heated plate set to 37°C to maintain temperature in a lamina flow hood. Every follicle that was 100µm in diameter or above was dissected out of the cortical strips using 25 gauge needles (Terumo Europe, Belgium) attached to a 1 ml syringe barrels (BD Plastipak, Becton Dickinson and Company, Madrid, Spain), ensuring as much stromal tissue was removed from around the ovarian follicle as possible without damaging it. The ovarian follicles were then cultured individually for a further 6 day in a humidified incubator at 37°C with 5% CO_2 in 96 well v-bottomed plates (Corning Co-star Europe, Badhoevedorp, The Netherlands), with one follicle per each well in 150µl bovine McCoy's medium with the addition of 100ng/ml recombinant human Activin A (R&D Systems, Abingdon, UK). The medium was changed every 48 hours by removing 75µl from the well and replacing it with 75µl of fresh bovine McCoy's medium with recombinant human Activin A.

The diameter of each follicle was measured immediately after dissection using a light microscope and then every 48 hours alongside the medium changes, at

two perpendicular planes. After a further 6 days in culture the individual follicles were fixed in formalin, processed and placed onto slides for H&E staining (see chapter two for full details).

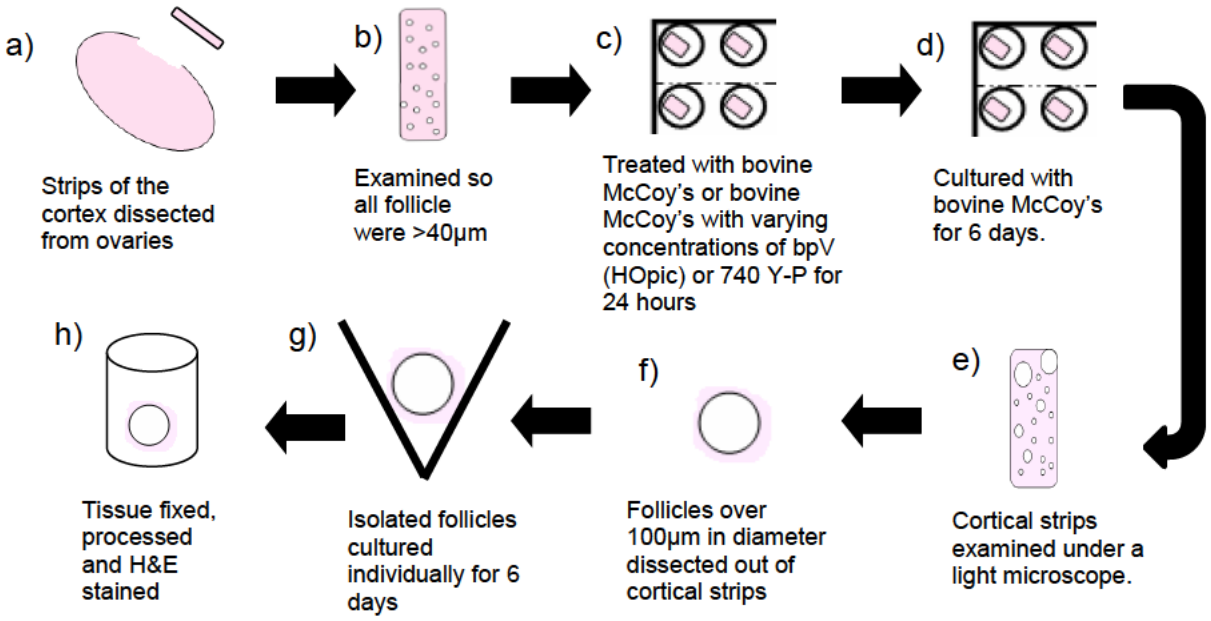


Figure 6.2: Tissue Culture Method. a) Strips of the cortex were taken from the bovine ovary and b) examined under a light microscope to ensure no follicles were 40µm or larger in diameter. c) Cortical strips were treated with bovine McCoy's or bovine McCoy's with the varying concentrations of bpV (HOpic) or 740 Y-P for 24 hours and d) further cultured in bovine McCoy's only for a further 6 days. After 6 days the cortical strips were e) examined under a light microscope and f) any follicle that were 100µm or larger in size were dissected out of the cortical strips. These follicles were then g) further cultured for 6 days before being h) fixed, processed and H&E stained for analysis.

6.2.2 Analysis

The individually cultured follicles were H&E stained for analysis based on their morphology. Each ovarian follicle was measured in the section where the nucleolus was present, or if there was no nucleolus, the section containing the largest cross-section of the oocyte. Follicle diameters were taken twice at perpendicular angles with the average follicle diameter being calculated from these two measurements. The health of the ovarian follicle was based on (1) general circularity of the oocyte, (2) integrity of the oolemma in the oocyte, (3) presence of a germinal vesicle and nucleolus in the oocyte and (4) that at least 85% of the granulosa cells

were healthy. If these criteria were met the ovarian follicle was classified as healthy, and if not they were deemed unhealthy.

6.2.3 Statistical Analysis

The Kolmogorov Smirnoff test was used to test for normal distribution; further statistical tests were chosen based on whether the data was normal distributed or not. The bovine culture was repeated 7 times for the different treatments of both bpV (HOpic) and 740 Y-P. The proportion of the follicles collected from cultured cortical strips from the different treatment groups was compared using a chi-square test (n=7). The diameter of the isolated follicle at dissection and during the further 6 days of culture was compared between the control with the various concentrations of bpV (HOpic) or with the various concentrations of 740 Y-P using a one-way ANOVA, where this test reported a significant difference the groups were compared individually using a post hoc Tukey test. The health of the isolated follicles was compared using a chi-square test (n=7). The comparison of change in follicle size after treatments with various amounts of either bpV (HOpic) or 740 Y-P were compared to the control using a two-way ANOVA, where this test reported a significant difference the groups were compared individually using a post hoc Tukey test. Only p-values that were <0.05 were considered to be significant.

6.3 Results

6.3.1 The Proportion of Isolated Follicles Collected After Treatment with bpV (HOpic) or 740 Y-P.

Table 6.1 displays the number of secondary follicles obtained from each treatment group. Figure 6.3 shows the proportion of follicles obtained from each treatment group from the total number of follicles obtained in each experiment. The proportion of isolated follicles collected from the different treatment groups were compared against one another. No significant difference was observed in the proportion of isolated follicles collected from the control or bpV (HOpic) treatment groups with 37.8±3.0%, 18.8±4.1% and 15.6± 4.8% isolated from 1μM, 10μM and 100μM of bpV (HOpic) compared to 27.8±4.5% collected from the control (n=7) ($p>0.05$) (see figure 6.3 A). No significant difference was seen in the proportion of isolated follicles collected from the 740 Y-P treatment groups at 18.4±3.7%, 20.7±3.5%, 27.4±3.5% and 15.3±4.5% of the isolated follicles collected from 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml 740 Y-P respectively compared to 18.2±3.2% in the control (n=17) ($p>0.05$) (see figure 6.3 B).

Table 6.1: Number of secondary follicles collected from each treatment group.

	Treatments	No. Follicles
bpV (HOpic)	Control	18
	1μM	24
	10μM	15
	100μM	12
740 Y-P	Control	17
	0.1μg/ml	16
	1μg/ml	18
	10μg/ml	22
	100μg/ml	15

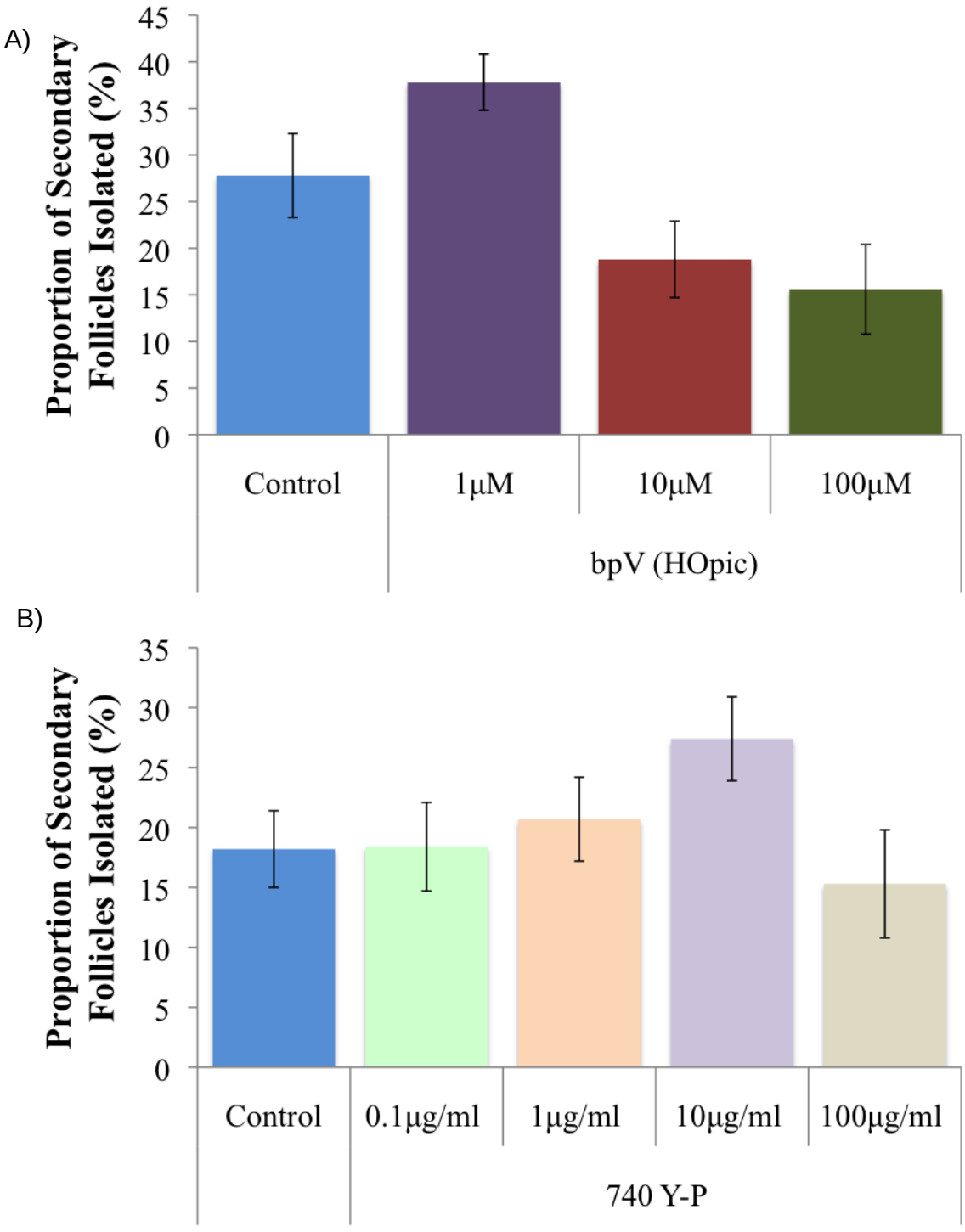


Figure 6.3: Proportion of Follicles Isolated from the Different Treatment Groups. These graphs display the proportion of large ovarian follicles isolated from the control group compared to either the A) bpV (HOpic) treatment groups or the b) 740 Y-P treatment groups. No significant difference was observed in the number of large follicles isolated from the control group in comparison to the a) bpV (HOpic) ($p>0.05$) treatment groups or the B) 740 Y-P treatment groups ($p>0.05$).

6.3.2 The Diameters of the Isolated Follicles after Treatment with bpV (HOpic) or 740 Y-P.

The diameters of the large ovarian follicles isolated from the cortical strips were measured directly after isolation prior to further culturing in the second step of the two-step culture system. No significant difference was observed between the mean diameter of the follicles isolated from the control (n=18) at 104.8μm ±3.8 and those treated with bpV (HOpic) which had mean diameters of 107.3μm ±3.2, 109.5μm ±3.9 and 104.3μm ±5.0 in 1μM (n=24), 10μM (n=15) and 100μM (n=12) of bpV (HOpic) ($p>0.05$) (see figure 6.4 A). No significant difference was observed in the mean follicle diameters of the follicles treated with 740 Y-P at 100.4μm ±4.4, 101.8μm ±4.6, 109.5μm ±3.4, 107.5μm ±3.4 in 0.1μg/ml (n=16), 1μg/ml (n=18), 10μg/ml (n=22) and 100μg/ml (n=15) 740 Y-P compared to the control (n=17) at 105.1 μm ±3.4 ($p>0.05$) (see figure 6.4 B).

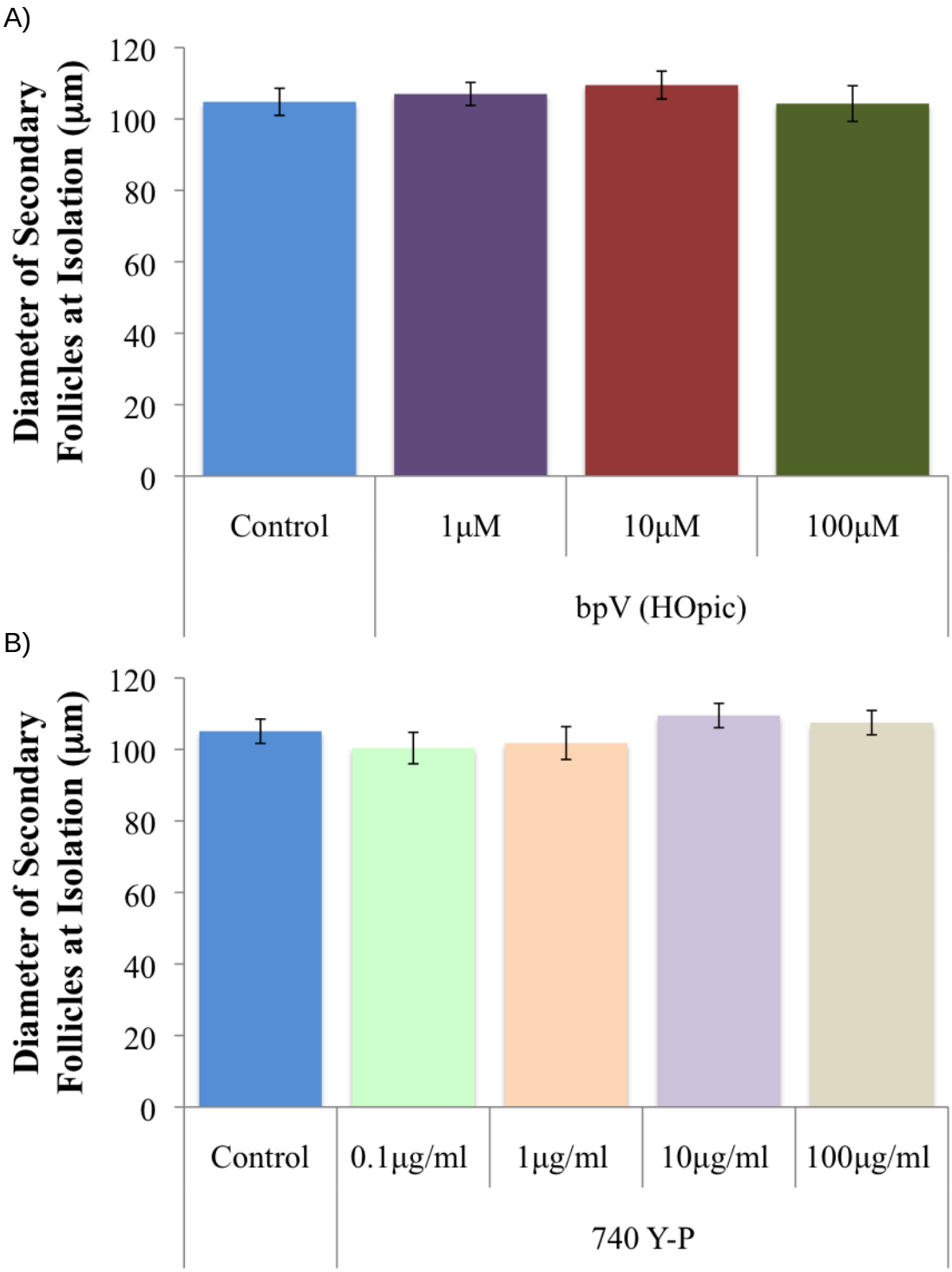


Figure 6.4: Mean Diameter of Large Ovarian Follicles at Isolation from the Different Treatment Groups. These graphs display the mean diameter of the ovarian follicles immediately after they were isolated after treatment with the control compared to either A) bpV (HOpic) or B) 740 Y-P. No significant difference was seen in the mean diameter of the isolate ovarian follicles after treatment with either bpV (HOpic) ($p>0.05$) or 740 Y-P ($p>0.05$).

6.3.3 The Mean Diameter of the Follicle in Culture after Treatment with bpV (HOpic) or 740 Y-P.

The diameters of the large ovarian follicles isolated from the cortical strips were measured at regular intervals during the second step of the two-step culture system. No significant difference was observed in the mean diameters of the isolated follicles during this culture period with either the various concentrations of bpV (HOpic) ($p>0.05$) or 740 Y-P ($p>0.05$) in comparison to the control at any time point (see figure 6.5). Although there was seen to be no significant difference in the change in follicle diameter over time both treatments with bpV (HOpic) and 740 Y-P displayed a non-significant trend towards the growth of the ovarian follicles stimulated with the higher concentrations having a lower growth rate with ANOVA $p<0.06$ for both.

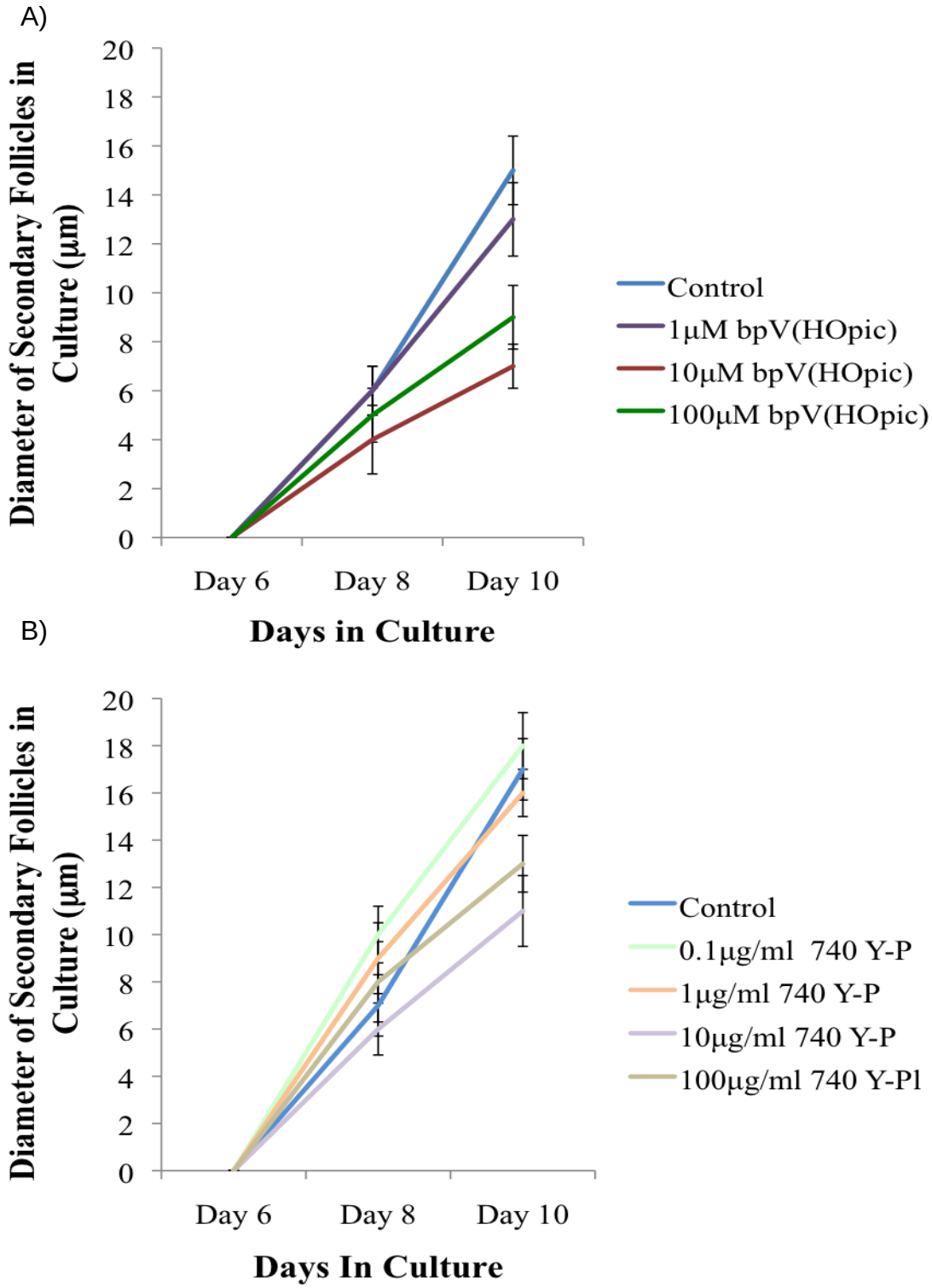


Figure 6.5: Mean Diameter of the Isolated Ovarian Follicles in Culture from the Different Treatments. These graphs show the change in the mean diameter of the isolated follicles during in the culture period in the control compared with A) bpV (HOpic) and B) 740 Y-P. Neither bpV (HOpic) ($p>0.05$) nor 740 Y-P ($p>0.05$), displayed a significant difference in mean follicle diameter during in the culture period.

6.3.4 The Follicular Health of the Isolated Follicles after Treatment with bpV (HOpic) or 740 Y-P.

The health of the isolated ovarian follicles was examined at the end of the culture period. The health of the isolated follicles that has been treated with bpV (HOpic) displayed no significant difference in health at 51.0±4.8%, 48.7±4.3%, 46.0±6.4% in 1μM, 10μM and 100μM of bpV (HOpic) compared to the control at 54.3±4.0% (n=7) ($p>0.05$) (see figure 6.6 A). Similarly, no significant difference was observed in the health of the isolated follicles after treatment with 740 Y-P at 52.2±3.8%, 54.8±3.84%, 50.7±4.9%, 48.6±4.3% 0.1μg/ml, 1μg/ml, 10μg/ml and 100μg/ml in comparison to 55.5±3.7% in the control (see figure 6.6 B). Although there is no significant difference there is a trend towards an increase in the concentration of with both bpV (HOpic) and 740 Y-P resulting in a decrease in the health of the isolated follicles.

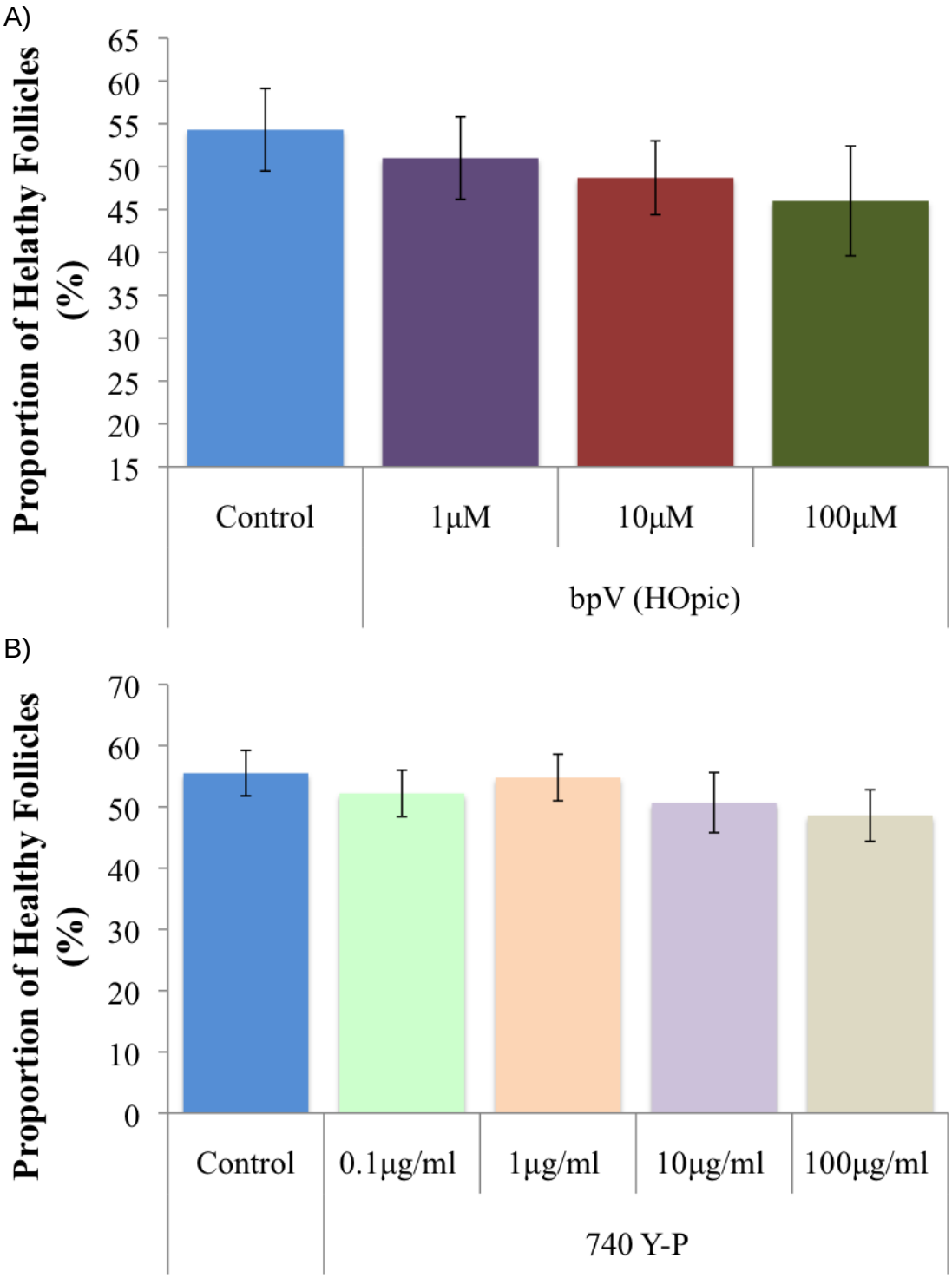


Figure 6.6 Proportion of Healthy of Ovarian Follicles. These graphs display the proportion of healthy ovarian follicles after culture with A) bpV (HOpic) or B) 740 Y-P in comparison to the control. No significant difference was observed in the proportion of ovarian follicles after treatment with the control in comparison to bpV (HOpic) (n=7) ($p>0.05$) or 740 Y-P (n=7) ($p>0.05$).

6.4 Discussion.

The aim of this study was to further examine if the increased primordial follicle activation via the PI3K pathway had any impact on the later stages of folliculogenesis. This was achieved by using the pharmacological compounds bpV (HOpic) and 740 Y-P in an *in vitro* bovine model, using the two step culture system developed by Telfer et al., 2008 and McLaughlin and Telfer, 2010, to stimulate the PI3K pathway and cause an increase in primordial follicle activation and then examine the follicles development at the secondary stage of folliculogenesis. The results display that the number of large follicles obtained did not change after treatment with either bpV (HOpic) or 740 Y-P. The results also showed that development and the health of the secondary follicles by the initial treatment with bpV (HOpic) or 740 Y-P was the same as those from the control group.

The PI3K pathway has been shown to play an important role in controlling primordial follicle activation (Castrillon et al., 2003; John et al., 2008; Li et al., 2010; Liu et al., 2007; McLaughlin et al., 2014; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008), with the up-regulation of the PI3K pathway causing increased activation. Chapter 5 showed that an up-regulation of the PI3K pathway caused increase follicle activation and therefore, an increased proportion of primary follicles. Despite this increase in activation there was no significant increase observed in the number of large ovarian follicles obtained after treatment with either bpV (HOpic) or 740 Y-P. This result differs from previous mouse and the human studies where there was an increase in the number of further developed follicles (Castrillon et al., 2003; Li et al., 2010; McLaughlin et al., 2014; Rajareddy et al., 2007; Reddy et al., 2008). It is possible that the secondary follicles need more time to develop from the primary follicle population as follicular development is known to be a protracted process (Adams and Pierson, 1995) and perhaps if the cortical strips had been cultured for a longer period more secondary follicles would have develop. However, there are other factors that could be influencing the proportion of secondary follicles produced.

One factor influencing the number of large ovarian follicles obtained after treatment with bpV (HOpic) or 740 Y-P could be the due to the observed decrease in the health of the primary follicle population seen in chapter 5. The quality of an

ovarian follicle and its oocyte is a key factor in determining if an ovarian follicle will continue through folliculogenesis (Blondin and Sirard, 1995; Hendriksen et al., 2004; Salamone et al., 1999). With the dramatic decrease in the health of the primary follicles seen in the chapter 5, it is possible to hypothesis that the increase in degenerative primary follicles will result in many of the follicles undergoing atresia at the primary follicle stage rather than continuing to develop through the progressive stages of folliculogenesis.

Despite the fact there is seen to be an increase in the number of primordial follicles being activated there was no observed increase in the proportion of secondary follicles in chapter 5 or large follicle in chapter 6. The decrease in the morphological health of the secondary follicles in study by McLaughlin et al., 2014 in the human and a trend towards a decrease in secondary follicles health observed within this thesis, indicates that there would not be an increase in the number of mature oocytes produced. Therefore, this chapter and previous study indicate that up-regulating activation via the PI3K pathway does not necessarily cause an increase in the number of mature oocyte obtained. Primordial follicles are activated in small cohorts that decrease in number as they progress through the various stages of folliculogenesis due to loss by atresia (Fortune, 1994; Macklon and Fauser, 1999 Morbeck et al., 1992). Only a set number of primary follicles develop into secondary follicles to control and ensure the correct number of dominant follicles are produced dependent on the species therefore, only allowing a controlled number of primary follicles to reach the secondary stage of follicle development.

The ovarian follicles isolated at day 6 were similar in diameter in each treatment group to the control group, despite chapter 5 showing that the up-regulation of the PI3K pathway causing an increase in the diameter of the primary follicles. The results from chapter 5 conveyed that the enlargement of the ovarian follicles was seen to coincide with a decrease in the health of the follicles, which is thought to be due to a disruption in the communication between the oocyte and its granulosa cells, which is essential to maintain the health of the ovarian follicles (Elvin et al., 2000; Fair, 2010; Moor, 1988; Nagyova et al., 2000; Rodgers et al., 2000; van den Hurk and Zhao, 2005). Therefore, it is likely that many of the primary

follicles were unable to develop to the later stages of folliculogenesis due to this increase in the proportion that became degenerative.

In summary, the PI3K pathway in the bovine model has only been observed in this these to impact the development of the ovarian follicles at the activation of the quiescent to primary follicles stage of development, but not later stages of follicular developmental. The results of this thesis confirms the results of the previous studies by Castrillon et al., 2003 and John et al., 2007 in the mouse model that also showed that the role of the PI3K pathway in folliculogenesis is limited to the early stages of folliculogenesis (Castrillon et al., 2003; John et al., 2007). The role of the PI3K pathway in folliculogenesis is restricted to the first stage of folliculogenesis as its key downstream components, Foxo3a and p27, are only active in the nucleus where they inhibit the quiescent follicles development. When the PI3K pathway is stimulated these components are exported into the cytoplasm where they are degraded allowing quiescent follicles to become primary follicles (Blume-Jensen and Hunter, 2001; Brunet et al., 2001; Cantley, 2002; Engelman et al., 2006; Stokoe, 2005; Vanderhyden, 2002, Accili and Arden, 2004; Arden and Biggs, 2002; Liu et al., 2007, Fero et al., 1996; Rajareddy et al., 2007; Zhang et al., 1999). The degradation of these key components after activation means that they will be unable to influence the later stages of follicular growth as observed in this study. Therefore, although the PI3K pathway is known to play an important role in cellular growth (Cantley, 2002; Stokoe, 2005) this role is limited to the activation of the quiescent follicles into primary follicles and does not impact the growth of the large isolated ovarian follicles cultured within this chapter. Subsequently, there was no increase in the number or growth of these large ovarian follicles this chapter indicating that the increase in activation of the quiescent follicles via the PI3K pathway does not cause an increase in the number of secondary follicles and therefore, it is unlikely that more mature oocytes would be produced.

Chapter Seven:
General Discussion

7.1 General Discussion

It is widely accepted that in most mammalian species the only source of oocytes that have the potential to mature and be fertilised is produced in fetal or early life, with no additional primordial follicles being created at any other point during the female's life (McGee and Hsueh, 2000; Zuckerman, 1951). Oocytes are held in an immature state in primordial follicles (Escobar et al., 2011; McGee and Hsueh, 2000). In order for a mature oocyte to be produced the primordial follicles must be activated to undergo folliculogenesis (Binelli and Murphy, 2010; McGee and Hsueh, 2000). There is still much we do not know about quiescent follicle activation, and although there is evidence of a number of different factors that are known to play a role, the mechanisms involved are still unclear (Adhikari et al., 2013; Kim, 2012; Monget et al., 2012; Pangas, 2012; Reddy et al., 2010). The PI3K pathway has been shown to be a key factor in the activation of primordial follicles (Castrillon et al., 2003; John et al., 2008; Li et al., 2010; Liu et al., 2007; McLaughlin et al., 2014; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008). However, the role of the PI3K pathway has been primarily explored in the rodent model therefore, it is important to explore its role in other species to see if it has the same central role.

The main aims of this thesis were to therefore; (1) to explore if an increase in the stimulation of the PI3K pathway impacts primordial follicle activation, health and subsequent growth in the bovine model. (2) Examine if increased stimulation of the PI3K pathway caused any changes in the development of the activated follicles. It was also important to (3) explore the location and distribution of the ovarian follicle populations, including the primordial follicles, within the ovary and to examine if their location within the ovary had any influenced on health of the ovarian follicle populations. Lastly, to (4) examine if the bovine is a good model of human primordial follicle activation and subsequent growth within an *in vitro* culture system.

The ovary consists of three main components; the surface epithelium, the cortex and the medulla (Escobar et al., 2011). Analysis of the bovine ovarian follicle populations (chapter three) and other studies showed that the ovarian follicle population is concentrated in the cortex of the ovary. The distribution of the different developmental stages of the ovarian follicles was different within different regions of

the ovary. The quiescent follicles population was concentrated in the outer region of the ovary whereas, a larger proportion of the ovarian follicle populations were growing follicles in the progressively deeper regions of the ovary (Jimenez, 2010; vanWezel and Rodgers, 1996). This change in the distribution of the different developmental stages of the ovarian follicles in the different regions of the ovary could be due to the timing of ovarian follicle formation. The first primordial follicles are formed in the medulla and they are thought to be the first to be activated and therefore, first to be lost (Henderson and Edwards, 1968; Hirshfield, 1991; Mork et al., 2012). A previous study in the mouse indicated that the timing of follicle formation influenced the ability of the ovarian follicles to maintain their dormancy, due to the progenitors not having fully developed in those formed earlier (Mork et al. 2012). The lower concentration of ovarian follicles in the medulla and cortex-medulla of bovine and human could be an indication that something similar happens in these two species, although further investigation is required to establish this. The line hypothesis (Henderson and Edwards, 1968), the progenitor's development (Mork et al. 2012) and the Kristensen et al., 2010 study all indicate that age is a key factor to the presence of primordial follicles within the medulla. One criticism of the present study is that a wider variety of ages should have been used, as it would have given a better insight to what happens to the ovarian follicles within the medulla. It is possible to speculate that ovarian follicles were found in the medulla of the bovine and not in the human in this study, because the bovine ovarian tissue samples were from comparatively younger animal than the human ovarian tissue samples.

The observed decrease in health of follicles located in the cortex and medulla could also have contributed to the lower concentration of ovarian follicles in the inner regions of the ovaries. This decrease in health appears to be due to the different environments surrounding the ovarian follicles, as there was no difference in the health of the ovarian follicles from the outer cortex and inner cortex in the bovine model. This decrease in health could be due to the concentration of the stromal cells surrounding the ovarian follicles, as cortex of the ovary that has a higher concentration of stromal cells had proportionally more healthy follicles compared to the cortex-medulla and medulla where the stromal cells are less concentrated. The role of the stromal cells in maintaining the health of ovarian follicles is unclear

however, the stroma is known to induce differentiation of the theca cells, which does not occur with medullary stromal cells (Orisaka et al., 2006) indicating that they are required in the process of folliculogenesis. However, further investigation is required to understand the role of the stromal cells in preserving follicular health. It would be interesting to examine if stromal environment required is different at the various stage of follicular development, as growing follicles are though to migrate from the outer cortex to the inner regions of the ovary (Woodruff and Shea, 2011). The data from the present study showed that the reduction in the distribution and health of the quiescent follicles in the inner regions of the ovary makes the cortex region a more favourable region to use for culture in functional studies exploring the quiescent follicle population and therefore, was the region utilised in the later experiments on the role of the PI3K pathway in primordial follicles activation.

The role of the PI3K pathway in this thesis was explored in the bovine. The bovine has similar attributes to the human in folliculogenesis (Adams and Pierson, 1995; Baerwald, 2009; Gougeon, 1996) making it a good candidate for this study as it would not only show the role of the PI3K pathway in the bovine, but also give a good indication of role of the PI3K pathway in the human. The rate of follicle activation and development was compared between the bovine and the human in an *in vitro* culture system (chapter 4). It was observed that the human and the bovine had similar levels of activation with a similar percentage of ovarian follicles becoming primary and secondary follicles. The level of growth in the transition between a quiescent follicle and a primary follicle was also shown to be similar in the two species. These similar attributes indicate that the bovine is a good model for primordial follicle activation *in vitro* for the human. For future studies it would be beneficial to further compare the bovine and human follicular development to include the later stages of folliculogenesis *in vitro* to see if the bovine model continues to be a good model for the human in later stages of folliculogenesis, as it would be useful to have a model that is similar to the human throughout the entire process of folliculogenesis.

The up-regulation of the PI3K pathway in the bovine model was shown to cause an increase in primordial follicle activation (chapter 5), agreeing with previous studies in the mouse (Castrillon et al., 2003; John et al., 2008; Li et al., 2010; Liu et

al., 2007; McLaughlin et al., 2014; Rajareddy et al., 2007; Reddy et al., 2009; Reddy et al., 2008). Interestingly the different concentrations of bpV (HOpic) and 740 Y-P did not result in different levels of activation, though it was shown to impact the health of the quiescent and primary follicle populations. However, it is possible to speculate that perhaps there is an upper limit on the number of quiescent follicles that can be activated at one time, which could be causing the decrease in the number of health quiescent and primary follicles. It is possible to hypothesis that utilisation of lower levels of stimulation of the PI3K pathway could have revealed that the level of activation of quiescent follicle is dose-dependent however, further investigation is required to explore this theory.

The PI3K pathway is known to play an important role in cellular growth (Cantley, 2002; Stokoe, 2005) and is seen to influence the growth of the oocytes, which become enlarged in the primary and quiescent follicles when the PI3K pathway is up regulated (chapter 5) agreeing with previous studies (Castrillon et al., 2003; John et al., 2008; Reddy et al., 2008). The enlargement of oocytes suggests that the communication between the oocyte and its granulosa cells has been disrupted, which is particularly highlighted in the quiescent follicles where there are enlarged oocyte indicated that the oocyte has been activated to grow but the granulosa cells do not appear to have been stimulated to begin to proliferating, creating a population of activated transitory follicles. A lack of communication between the oocyte and its granulosa cells could be a key factor causing a reduction in the health of the bpV (HOpic) and 740 Y-P treated ovarian follicles, which must be balanced for the ovarian follicle to remain healthy (Elvin et al., 2000; Fair, 2010; Moor, 1988; Nagyova et al., 2000; Rodgers et al., 2000; van den Hurk and Zhao, 2005). Enlarged oocytes in the primary and quiescent follicles were observed in previous mouse studies (Castrillon et al., 2003; John et al., 2008; Reddy et al., 2008). Primary and quiescent follicle containing these enlarged oocytes did not appear to be able to progress to the later stages of folliculogenesis and eventually degenerated. The importance of maintaining balanced development between an oocyte and its ovarian follicle is highlighted in this thesis by a lower proportion of the healthy ovarian follicles being observed in the follicle diameters above the range seen in the control ovarian follicles (chapter 5). This imbalance of development between the oocyte and

its granulosa cells maybe because the PI3K pathway has a bigger influence on the development of the oocyte than the granulosa cells, indicated by oocyte-specific deletion of PTEN (Reddy et al., 2008) causing increased follicular activation, whereas, granulosa cell-specific deletion of PTEN (Fan et al., 2008) had no impact on the level of follicular activation.

Despite the increase in activation and therefore, increase in the number of primary follicles, the secondary follicle population was not seen to increase (chapter 5). Neither was there a significant increase in the number of large secondary follicles that were isolated during the second step of the two-step culture system after increased quiescent follicle activation via the up-regulation of the PI3K pathway (chapter 6). This lack of further developed ovarian follicles could be due to the decrease in proportion of healthy primary follicle seen in chapter 5, as the quality of the ovarian follicle is key in determining whether it will continue through folliculogenesis or undergo atresia (Blondin and Sirard, 1995; Hendriksen et al., 2004; Salamone et al., 1999). Therefore, the high level of degeneration exhibited in the primary follicle after treatment with bpV (HOpic) and 740 Y-P will mean that many of the primary follicles would have been unable to progress further in their development. However, the ovarian follicles that were able to progress to the later stages of folliculogenesis were seen to be similar in size and health to the control follicles when isolated at day 6 (chapter 6).

These results differ from the previous human study *in vitro* where the up-regulation of the PI3K pathway had no impact on the growth of either the quiescent or primary follicle populations and consequently the increase in activation resulted in an increase in the number of secondary follicle obtained (Li et al., 2010; McLaughlin et al., 2014). The difference between this thesis and the previous studies may be related to the comparative ages at which the ovarian samples were taken, as the bovine material has been derived from younger individuals and so has a high concentration of ovarian follicles, (McGee and Hsueh, 2000) as seen in chapter 4. This means that there was a larger number of quiescent follicles being activated in the bovine and consequently this will have cause more inhibitory factors to be produced as there are more growing follicle, which would inhibit follicle activation and growth of other ovarian follicles (Edwards et al., 1977; McGee and Hsueh, 2000).

This may be why the quiescent and primary follicles populations are seen to have a proportion decrease in health in the bovine model from younger tissue samples in chapter 5 in comparison to the human with older tissue sample utilised in the previous studies. The results presented in chapters 5 and 6 are most likely to be a good indication of what would occur in a younger human ovary. Despite this differences between the bovine and human the *in vitro* culture systems both display a decrease in the survival of the ovarian follicles after increased activation via the PI3K pathway, so there is little chance of more mature fertilisable oocytes being produced thorough increased stimulation of the PI3K pathway.

7.2 Concluding Remarks

This thesis aimed to explore the role of the PI3K pathway in a large mono-ovulatory species, using an *in vitro* bovine model. The up-regulation of the PI3K pathway was shown to cause an increase in the activation of the ovarian follicles. This thesis also identified that up-regulation of the PI3K pathway caused a decrease in the health of both the quiescent and primary follicle populations. This decrease in health was dose-dependent, with higher doses of bpV (HOpic) and 740 Y-P causing increased degeneration in the ovarian follicle populations. The up-regulation of the PI3K pathway resulted in the enlargement of the oocytes in the quiescent and primary follicles, which could be a contributing factor to the increased degeneration in the primary and quiescent follicle populations. As a result there was no significant increase in the number of further developed ovarian follicles and therefore, it is unlikely that more fertilisable oocytes would have been produced.

Recently, a live human birth has been reported using bpV (HOpic) and 740 Y-P treatments of ovarian fragments in vitro, followed by replacement and IVF (Kawamura et al., 2013). However, the study did not have a control and the ovary was cut up which could have an impact on other pathways as well as the PI3K pathway so it is hard to determine if this was due to the stimulation of the PI3K pathway. Chapter 5 showed that the utilisation of these pharmacological compounds in the bovine model resulted in a decrease in the health of not only the growing follicle populations, but also the quiescent follicle population. Chapter 5 also showed

there was no increase in follicular activation despite an increase in concentration of both bpV (HOpic) and 740 Y-P, although there is a decrease in the health with an increase in concentration. Therefore, it might be possible to find a concentration that causes the up-regulation of the PI3K pathway without causing a decreased in the health of the follicles. Further research is required to establish non-detrimental concentrations that can be safely used in clinical applications.

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